



**Exercise Nutrient Interactions: Maximising Training Adaptation and Performance**

A thesis submitted in fulfilment of the requirements for the Doctorate of Philosophy

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August 2014

**Declaration**

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis/project is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Stephen Lane

30/1/2015

## **Research Outcomes**

Publications arising from the work undertaken for this thesis:

### **Peer Reviewed Publications**

**Lane, S. C., Areta, J. L., Bird, S. R., Coffey, V. G., Burke, L. M., Desbrow, B., Hawley, J. A.** (2013b). Caffeine ingestion and cycling power output in a low or normal muscle glycogen state. *Med Sci Sports Exerc*, (*Impact Factor: 3.71*) 45(8), 1577-1584. doi: 10.1249/MSS.0b013e31828af183

**Lane, S. C., Bird, S. R., Burke, L. M., & Hawley, J. A.** (2013). Effect of a carbohydrate mouth rinse on simulated cycling time-trial performance commenced in a fed or fasted state. *Appl Physiol Nutr Metab* (*Impact Factor: 2.009*), 38(2), 134-139. doi: 10.1139/apnm-2012-0300

**Lane, S. C., Hawley, J. A., Desbrow, B., Jones, A. M., Blackwell, J. R., Ross, M. L., Zemski, A. J., Burke, LM.** (Accepted October 2013) Single and combined effects of beetroot juice and caffeine supplementation on cycling time trial performance. *Appl Physiol Nutr Metab* (*Impact Factor: 2.009*). <http://www.nrcresearchpress.com/doi/abs/10.1139%2Fapnm-2013-0336>

### **Manuscripts in Preparation**

**Lane, S. C.**, Camera, D. M., Areta, J. L., Bird, S. R., Burke, L. M., Jeacocke, N. A., Yeo, W. K., Hawley, J. A. The effects of delayed post-exercise feeding on acute metabolic responses and a subsequent exercise bout.

### **Conference Abstracts**

**Lane, S. C.**, Coffey, V. G., Areta, J. A., Bird, S. R., Hawley, J. A. The effects of caffeine ingestion on self-selected power output during cycle interval training commenced with normal or low muscle glycogen content. Oral presentation given at: European College of Sports Science Conference July 6 – 9 2011, Liverpool, UK.

**Lane, S.**, Hawley, J., Burke, L. Caffeine and beetroot juice: A bittersweet combination? Oral presentation given at: Sports Dietitians Australia Conference, October 18 –19 2013, Melbourne, Australia.

## ***Declaration***

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Signed:

Stephen C. Lane

Date: \_\_\_\_\_

## ***Acknowledgements***

- Firstly, I would like to thank my family, my mother Anne, my father Ian and my little sister Kerrie. Given my academic record prior to discovering my love for the human body, and in particular skeletal muscle, the journey life has taken me is a surprise to many I am sure, even myself. It was your love and encouragement that led me to open new doors and follow the path to where I am now. Without your support I would not have been able to follow my dream and feed my inquisitive mind. I am proud of what I have achieved, and knowing you feel the same way makes all the hard work worthwhile.
- I would like to thank all past and present members of the Exercise Nutrition Research Group that have at some stage provided me guidance and assistance throughout my duration at RMIT: Josè Arèta, Donny Camera, Evelyn Parr, Vernon Coffey, Will Smiles, Kristylen Tomcik and Erin Stephenson. Seeing that we all go through similar struggles and accomplishments make me feel as though this long path I have travelled has not been alone.
- Many thanks to Professor John Hawley for providing me the opportunity to pursue my PhD studies under your guidance. Little did I know during my undergraduate years, that I was lucky enough to be at the same university as one of the world's leading researchers in the field of exercise nutrient interactions. Your supervision, knowledge and positive outlook made my PhD experience something I will always fondly remember. I can only hope to one day attain the same amount of knowledge regarding exercise training adaptation, and the sensibility to practically apply it (and summate it into a single paragraph) as you do.

- Thank you to my primary supervisor Professor Stephen Bird. Over the years, and especially in the final and most difficult few months while finalising this thesis, you provided the support, guidance and an eye for detail that I needed to summate, and present my last four years of work.
- To all the subjects that volunteered for the studies undertaken during this thesis, many of which have now become close friends. I cannot thank you enough. Without your commitment, time and energy this work would not have been possible. I hope, armed with the findings of the projects from my thesis and the discoveries still to come I can one day help you go faster and achieve your optimal performance.
- Thank you Dr. Andrew Garnham for safely and professionally performing the muscle biopsies in study 2 of this thesis. Your skill and character nearly make the invasiveness of a biopsy a pleasure.
- I would like to thank the different funding sources for each of the studies in this thesis including the Australian Institute of Sport as well as the Australian postgraduate scholarship award program.

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## ***Abbreviations***

ACC	acetyl-Coenzyme A carboxylase
AMPK	5' adenosine monophosphate kinase
AT	aerobic training
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
BM	body mass
CAFF	caffeine
Ca <sup>2+</sup>	calcium
cDNA	complementary DNA
CD36	cluster of differentiation 36
CHO	carbohydrate
CNS	central nervous system
COX	cytochrome C oxidase
CPT1	carnitine palmitoyltransferase I
CV	coefficient of variation
DTT	dithiothreitol
dw	dry weight
EDTA	ethylene-diamine-tetra-acetic acid
ES	effect size
FABP	fatty acid binding protein
FST	fasted

FED	fed
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Glut4	glucose transporter 4
GS	glycogen synthase
GSK	glycogen synthase kinase
HCl	hydrochloric acid
HIT	high intensity interval training
HPLC	high-performance liquid chromatography
HSL	hormone sensitive lipase
HSP	heat shock protein
IL	interleukin
kJ	kilojoule
LOW	low muscle glycogen concentration
LPL	lipoprotein lipase
MAPK	mitogen activated protein kinase
mRNA	messenger ribose nucleic acid
NORM	normal muscle glycogen concentration
NO	nitric oxide
NO <sub>2</sub> <sup>-</sup>	nitrite
NO <sub>3</sub> <sup>-</sup>	nitrate
PDH	pyruvate dehydrogenase

PDK4	pyruvate dehydrogenase kinase 4
PGC-1 $\alpha$	peroxisome proliferator activated receptor gamma co-activator-1 alpha
PLB	placebo
PPAR	peroxisome proliferator activated receptor
PPO	peak power output
RPM	revolutions per minute
RT-PCR	real time-polymerase chain reaction
SD	standard deviation
SE	standard error
SEM	standard error mean
TBS	tris-buffered saline
TCA	tricarboxylic acid
TFAM	mitochondrial transcription factor A
TT	Time trial
UCP3	uncoupling protein 3
VEGF	vascular endothelial growth factor
VO <sub>2max</sub>	maximal oxygen uptake
VO <sub>2peak</sub>	peak oxygen uptake
yr	year
W	watts
W <sub>max</sub>	maximal power





## **Abstract**

Athletes and coaches are continually striving to uncover new methods to enhance sporting performance. Optimal performance during competition is largely determined by the summation of the acute responses evoked by each training bout, and the resulting accumulation of functional proteins that ultimately define training adaptation. Within this context, nutritional and ergogenic strategies are capable of; i) further enhancing training adaptation and thereby improving performance during competition, and ii) enhancing performance directly on the day of competition. When considering such strategies it is important to distinguish between the objective of a training programme, which is to maximise adaptation - preparation for performance, and the goals for competition, which are performance focused. Hence the optimal nutritional strategies for training and competition may differ.

In the context of maximising training adaption, the effect of commencing selected workouts with reduced carbohydrate (CHO) availability has gained attention from both the athletic and scientific community. Current research is beginning to provide new evidence on which to base recommendations for nutrition to enhance training adaptation. However, further research is required in order to develop clear guidelines for the optimisation of such training strategies that incorporate this 'train-low' approach while also addressing practical implications that arise from this recently developed training methodology.

In contrast, in the context of maximising performance during competition a large body of work has demonstrated that CHO ingestion prior to and during endurance events lasting several minutes to several hours has the capacity to improve performance. Similarly, the incorporation of certain ergogenic compounds that elicit their effect through metabolic or centrally-mediated actions has also been shown to enhance performance during a range of

endurance tasks. However, less is understood about the interactions between exercise, nutritional state and the efficacy of specific ergogenic aids under conditions of optimal or sub-optimal CHO availability.

The experimental work undertaken for this thesis examined: i) the effects of practical nutrition and exercise training strategies, specifically conditions of low CHO availability, on metabolic markers of endurance training adaptation, and ii) the efficacy of known ergogenic aids during ‘real world’ conditions of both optimal and sub-optimal CHO availability. The first two studies investigated interventions to promote training adaption while the final two studies focused on strategies to optimise performance during competition.

The first study (Chapter Two) of this thesis determined the effect of a low dose of caffeine on maximal self-selected power output during high intensity interval training (HIT) commenced with either normal (NORM) or low (LOW) muscle glycogen availability. The main purpose was to determine whether caffeine could restore the previously reported reduction in power output when undertaking HIT with reduced muscle glycogen concentration. Twelve endurance-trained cyclists/triathletes performed four experimental trials using a double-blind Latin square design. Muscle glycogen content was manipulated via exercise-diet interventions so that two experimental trials were commenced with LOW and two with NORM muscle glycogen availability. Sixty minutes prior to an experimental trial, subjects ingested a capsule containing anhydrous caffeine (CAFF; 3 mg<sup>-1</sup>·kg<sup>-1</sup> BM) or placebo (PLBO). Power output (W) was measured throughout a standardised bout of high-intensity interval training (HIT; 8 x 5 min bouts at maximum self-selected intensity with 1 min recovery). There were significant main effects for both pre-exercise glycogen content and caffeine ingestion on power output. LOW reduced power output by ~8% compared to NORM ( $P < 0.01$ ) whereas caffeine increased power output by 2.8% and 3.5% under NORM and LOW conditions

respectively ( $P < 0.01$ ). It was concluded that caffeine enhanced power output independently of muscle glycogen concentration but in conditions of LOW could not fully restore power output to levels commensurate with that when subjects commenced exercise with normal glycogen availability. However, the reported increase in power output does provide a likely performance benefit, and through the partial restoration of training intensity may offer a means to further enhance the already augmented training response observed when selected sessions are commenced with reduced muscle glycogen availability.

The second study, described in Chapter Three, investigated the effects on acute cellular responses when subjects undertake a bout of HIT in the evening and feeding is withheld during recovery, a protocol referred to as ‘train-high sleep-low’. This study also aimed to determine the acute cellular response of commencing a second aerobic training (AT) session with reduced glycogen availability after ‘sleeping low’. This order of exercise (HIT then AT) was used in place of the traditional ‘train-low’ model where an AT bout is used to deplete muscle glycogen followed by a subsequent HIT bout in which training intensity is compromised, due to the reduced CHO availability. The ‘train-high, sleep-low’ approach aimed to preserve overall training intensity by commencing HIT with normal or elevated muscle glycogen availability, and then restricting CHO feeding post-exercise so that subjects slept with low glycogen levels. The following morning a subsequent low intensity AT (~60%  $VO_{2peak}$ ) exercise bout was undertaken in the fasted state. By restricting feeding post HIT the signaling markers AMPK, p38MAPK and ACC were elevated the following morning compared to when CHO was ingested immediately post-exercise with no significant differences in gene responses evident at this time point. The subsequent AT exercise bout enhanced markers of fat transport and oxidation including the protein content of CPT1 and the mRNA abundance of CD36 and FABP to a greater extent in the FASTED compared to FED trial. However, there was no effect of commencing AT with reduced glycogen on

selected markers of mitochondrial biogenesis (including PGC1 $\alpha$  and Tfam). This study provides novel insight into the molecular responses of a ‘train-high, sleep-low’ nutritional and exercise training strategy aimed to augment acute training responses. This ‘train-high, sleep-low’ model when incorporated into a periodised training programme may serve as an additional stimulus to enhance rates of fat oxidation during a subsequent exercise bout. However, as a result of the lack in upregulation of mitochondrial markers in this study, it is suggested that future investigations should aim to determine if there is an absolute critical limit for glycogen concentration, as well as a threshold for exercise intensity that stimulates the previously observed enhanced mitochondrial adaptive response when exercise is commenced with reduced glycogen availability.

The third study (Chapter Four), determined the effects of a CHO mouth rinse on a simulated high-intensity cycling time trial commenced in either a fed or fasted state. Twelve competitive male cyclists each completed four experimental trials using a double blind Latin square design. Two trials were commenced 2 h after a meal containing 2.5 g·kg<sup>-1</sup> BM of CHO (FED) and two after an overnight fast (FST). Prior to, and after every 12.5% of total time during a performance ride, either a 10% maltodextrin (CHO) or a taste matched placebo (PLB) solution was mouth rinsed for 10 s then immediately ‘spat’ out. There were significant main effects for both pre-ride nutritional status (FED vs FST;  $P < 0.01$ ) and CHO mouth rinse (CHO vs PLB;  $P < 0.01$ ) on power output with an interaction between the interventions ( $P < 0.05$ ). The CHO mouth rinse improved mean power to a greater extent after an overnight fast (282 vs 273 W, 3.4%) compared to a fed state (286 vs 281 W, 1.8%). It was concluded that a CHO mouth rinse improves performance to a greater extent in a fasted compared to a fed state; however, optimal performance was achieved in a fed state with the addition of a CHO mouth rinse.

The final study, described in Chapter five, investigated the independent and combined effects of caffeine and nitrate ( $\text{NO}_3^-$ ) supplementation on the performance of a cycling task simulating the physical challenges of the London 2012 Olympic Games road cycling time trial (TT). These effects were investigated against the background of standardised dietary preparation strategies that are typical of time trial specialists including a CHO-rich pre-event meal; the ingestion of a CHO-electrolyte drink; and regular oral CHO contact during the TT. Twelve male and 12 female competitive cyclists each completed four experimental trials in a double blind Latin square design. Trials were undertaken with a caffeinated gum (CAFF;  $3 \text{ mg}\cdot\text{kg}^{-1}$  body mass [BM], 40 min prior to the TT), concentrated beetroot juice supplementation (BJ;  $8.4 \text{ mM}$  of  $\text{NO}_3^-$ , 2 hours pre-TT), caffeine plus beetroot juice (CAFF+BJ) or a control trial (CONT). Subjects completed the TT (Females: 29.35 km; Males: 43.83 km) on a laboratory cycle ergometer with direction to complete the set distance as quickly as possible. Compared to CONT, power output was significantly enhanced after CAFF+BJ and CAFF (3.0% and 3.9% respectively). There was no effect of BJ supplementation when used alone ( $-0.4\%$ ,  $P = 0.6$ ; compared to CONT) or combined with caffeine ( $-0.9\%$ ,  $P = 0.4$ ; compared CAFF). The results demonstrated that caffeine administered in the form a caffeinated gum increased cycling TT performance lasting ~50-60 min by ~3-4% in both males and females. Beetroot juice supplementation was not ergogenic under the conditions of this study.

Taken collectively, the results from the studies undertaken for this thesis provide practical strategies to enhance endurance training adaptation and performance under conditions of reduced and of optimal CHO availability. Specifically, the findings suggest that ingestion of low doses of caffeine ( $3 \text{ mg}\cdot\text{kg}^{-1}$  BM) prior to high intensity cycling improves self-selected maximal power output regardless of muscle glycogen availability. Additionally, a CHO mouth rinse was demonstrated to be effective in both a post-prandial and fasted state, with

optimal performance being observed after a pre-event meal in combination with a CHO mouth rinse. Collectively, the ergogenic effects of caffeine as well as the centrally mediated actions of a CHO mouth rinse under conditions of reduced CHO availability suggest a means to partially restore maximal self-selected training intensity under conditions of reduced glycogen concentration. The incorporation of these ergogenic supplements during ‘train-low’ interventions where intensity is likely to be compromised may enhance the previously observed augmented training response. Findings also suggest that during competition, the ergogenic supplements investigated provide a means to further enhance performance when athletes follow current sports nutrition guidelines.

This thesis also provides novel findings that the manipulation of daily feeding patterns in relation to exercise training sessions can augment the adaptive stimulus during recovery and subsequent exercise. In particular an approach where athletes complete intense training sessions late in the evening, ‘sleep-low’ and then complete a subsequent low intensity exercise bout the following morning with reduced muscle glycogen content, augments specific energy sensitive signaling kinases and upregulates genes with putative roles in lipid metabolism. These findings further enhance current knowledge in the practical application of training strategies that reduce CHO availability with the intent to promote training adaptation.

In summary, the findings of this thesis have provided evidence for strategies to: i) promote training adaptation, and ii) enhance performance under conditions of both high and low CHO availability. Specifically, the use of ergogenic aids to restore/enhance intensity during exercise training when CHO is intentionally reduced as well as during competition under conditions of optimal CHO availability. Lastly, this thesis provides evidence for a novel ‘train-high, sleep-low’ approach that sustains the intensity of training during HIT while still

affording a strategy to achieve the adaptive benefits of reduced CHO availability during recovery and subsequent exercise.





# ***Chapter One***

## ***Literature Review***

## **1.1 Introduction**

Athletes undertake exercise training to promote physiological adaptation with the objective of attaining peak performance during competition. During specific phases of training, the primary goal is to optimise the adaptive response with less emphasis placed on performance. Conversely, during competition, the main objective is to ensure an athlete's best performance is achieved. It is clear that high CHO availability during training and competition is essential to promote optimal performance. However, during training when adaptation is the primary goal the manipulation of diet (e.g. CHO availability) can serve as a means to alter the adaptive response.

Commencing selected training sessions with reduced CHO availability (eg. muscle glycogen) has been shown to improve both acute and chronic physiological markers of endurance training adaptation. However, such interventions have also been shown to reduce the ability to perform high intensity exercise during training and competition. With regard to training adaption, CHO availability has been manipulated in several ways; i) chronically low CHO diets, ii) training in an overnight fasted state, iii) withholding CHO intake during training, iv) training twice a day so that a second session is commenced with low endogenous CHO availability and v) withholding CHO during recovery from exercise. In the context of competitive sport, most elite endurance athletes with high training loads probably undertake a variety of these scenarios during their training with few using them as pre-planned strategies. In this regard, little research has been undertaken to investigate the physiological and performance outcomes of these strategies when incorporated into a periodised training programme: evidence-based research is needed if these practices are to be recommended for incorporation into the training regimes of athletes.

In pursuit of scientific evidence many studies have investigated a myriad of supplements that are purported to enhance sporting performance. Many of these ergogenics are used during competition in an effort to enhance performance. The use of ergogenic strategies during training is somewhat less well studied despite the possibility that adaptation may be enhanced if training intensity can be increased. Within this context, the efficacy and modes of action for many commonly used ergogenic aids suggest that their incorporation during training when intensity may be compromised (i.e by a lack of fuel substrate for working muscles) may be able to restore intensity and thereby improve the training stimulus. However, little research has directly investigated the efficacy of commonly used ergogenic supplements under conditions of both sub-optimal and optimal CHO availability. Therefore, further investigation into the effects of nutritional state on the efficacy of various ergogenic aids is warranted

This literature review will firstly focus on current strategies to optimise *training adaptation* through the manipulation of muscle glycogen content. Secondly, it will detail ergogenic strategies to enhance *performance* under conditions of either optimal or sub-optimal CHO availability. This background will provide the context for the studies that investigate the effects of nutritional interventions on training adaption as well as the premise for the incorporation of ergogenic aids during training and competition to promote performance.

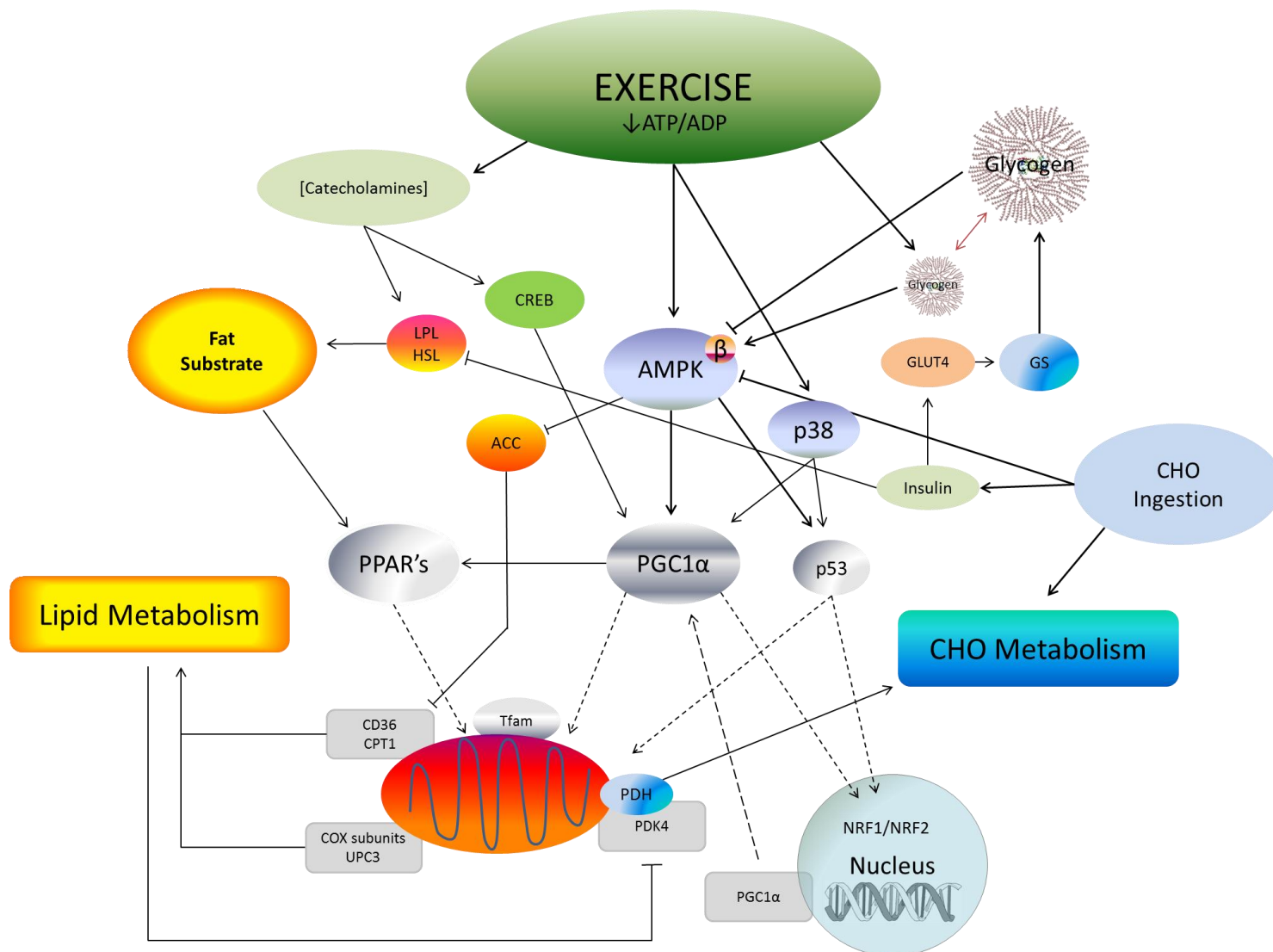
## ***1.2 Muscle glycogen as a signal for adaptation***

Skeletal muscle has the ability to undergo both structural and physiological adaptation in response to a range of external stimuli. This plasticity serves as a mechanism that enables skeletal muscle to adapt and better maintain homeostasis during subsequent exposure to stressors (i.e. exercise) (Coffey and Hawley, 2007). These primary stimuli include an array of

mechanical and biochemical perturbations including cellular ion status, hypoxia and energy status (Coffey and Hawley, 2007). While it has long been accepted that these primary stimuli are responsible for evoking specific adaptive responses it has only more recently been accepted that whole body and skeletal muscle substrate availability plays a putative role in the adaptation process (Hawley et al., 2011). Muscle glycogen is now considered to be not only a substrate for muscle contraction but also a recognised stimulus in the complex signaling events that result in the adaptation of skeletal muscle toward an endurance phenotype (Hawley 2013, Philp et al., 2012, Philp et al., 2011, Hawley and Morton, 2013).

### *1.2.1 The 5' adenosine monophosphate-activated protein kinase – A role in glycogen sensing*

Skeletal muscle glycogen concentration has been shown to influence specific signaling mechanisms sensitive to cellular energy status. The primary enzyme sensitive to muscle glycogen content appears to be 5' adenosine monophosphate-activated protein kinase (AMPK) (Winder, 2001). AMPK contains a  $\beta$  subunit that allows the binding of the kinase to glycogen (Hudson et al., 2003). As such, when glycogen stores are high AMPK activity is allosterically inhibited and conversely, when glycogen is less abundant, the allosteric inhibition of AMPK is reduced and subsequently more sensitive to phosphorylation (McBride et al., 2009). Exercise-induced glycogen depletion has been shown to activate AMPK in both rats (Philp et al., 2013) and humans (McBride and Hardie, 2009, Yeo et al., 2010, Chen et al., 2003) resulting in the activation of its downstream targets. AMPK also plays a role in fatty acid oxidation, glucose uptake and glycolysis (Winder, 2001, Wojtaszewski et al., 2003), as well as the stimulation and regulation of mitochondrial biogenesis through the direct activation of peroxisome proliferator activated receptor gamma co-activator-1 $\alpha$  (PGC1 $\alpha$ ) (Jager et al., 2007) (See Figure 1.1). The continual cycling of muscle glycogen via repeated exercise training and nutritional replenishment plays a pivotal role in the acute transient signaling response to endurance training. Much research has focused on the manipulation of CHO availability using exercise and nutritional interventions with the underlying hypothesis that this nutrient sensitive pathway may augment acute cellular responses that when combined into a periodised training programme may result in superior training adaptation (Hansen et al., 2005, Hulston et al., 2010, Morton et al., 2009, Yeo et al., 2008b).



**Figure 1.1.** Schematic overview of potential cell signaling pathways with roles in regulating mitochondrial adaptations when commencing endurance exercise with reduced muscle glycogen content. The reduction in muscle glycogen availability increases both 5' adenosine monophosphate-activated protein kinase (AMPK) and p38 mitogen activated protein kinase (p38MAPK) phosphorylation with subsequent increases in peroxisome proliferator activated receptor gamma co-activator-1 $\alpha$  (PGC-1  $\alpha$ ) activation and translocation the cell nucleus. In the nucleus, PGC-1 $\alpha$  co-activates nuclear regulatory factors to increase the expression of cytochrome C oxidase (COX) subunits and mitochondrial transcription factor A (Tfam) as well as auto-regulating its own expression. In the mitochondria, PGC-1 $\alpha$  co-activates Tfam to co-ordinate regulation of mtDNA and induces the expression of key mitochondrial proteins of the electron transport chain (i.e. COX subunits). The exercise induced elevations in catecholamine promote elevations in free fatty acids through hormone sensitive lipase (HSL) and lipoprotein lipase (LPL) which subsequently upregulate the nuclear transcription factor, peroxisome proliferator activated receptor  $\delta$  (PPAR $\delta$ ), to increase expression of key proteins involved in regulation of lipid oxidation such as carnitine palmitoyltransferase I (CPT-1), pyruvate dehydrogenase kinase 4 (PDK4) and cluster of differentiation 36 (CD36). CHO ingestion reduces lipid metabolism via insulin secretion and suppresses mitochondrial gene expression via the reduction in AMPK activity. Solid lines represent activation/deactivation; dashed lines represent translocation (Figure adapted from Hawley and Morton 2013).



### *1.2.2 Amplifying the acute training response*

Chan and colleagues (2004) were one of the first groups to examine the role of commencing exercise with different glycogen concentrations. In that study muscle glycogen was manipulated through either a normal or low CHO diet prior to 60 min of submaximal exercise. These workers reported that exercise commenced with reduced glycogen availability increased phosphorylation of nuclear p38 MAPK compared to when the same exercise bout was commenced with normal glycogen stores (Chan et al., 2004). Similarly, Wojtaszewski et al., (2003) had a group of subjects complete two experimental trials of 60 min cycling at 70%  $\text{VO}_{2\text{peak}}$  with either high or low glycogen concentrations ( $\sim 900$  vs  $\sim 160$   $\text{mM}\cdot\text{kg}^{-1}$  BM dry weight). In that study glycogen was manipulated by a prior depleting exercise bout and the restoration of glycogen in the high trial using a high CHO diet prior to the subsequent experimental exercise bout. These researchers reported AMPK activity in resting skeletal muscle and the degree of activation during exercise was increased under conditions of low glycogen concentrations despite no differences in muscle high-energy phosphates [AMP/ATP], creatine or creatine phosphate concentrations. Wojtaszewski et al., (2003) also reported that AMPK activity may be regulated by substrate availability and elevations in humoral factors such as catecholamines.

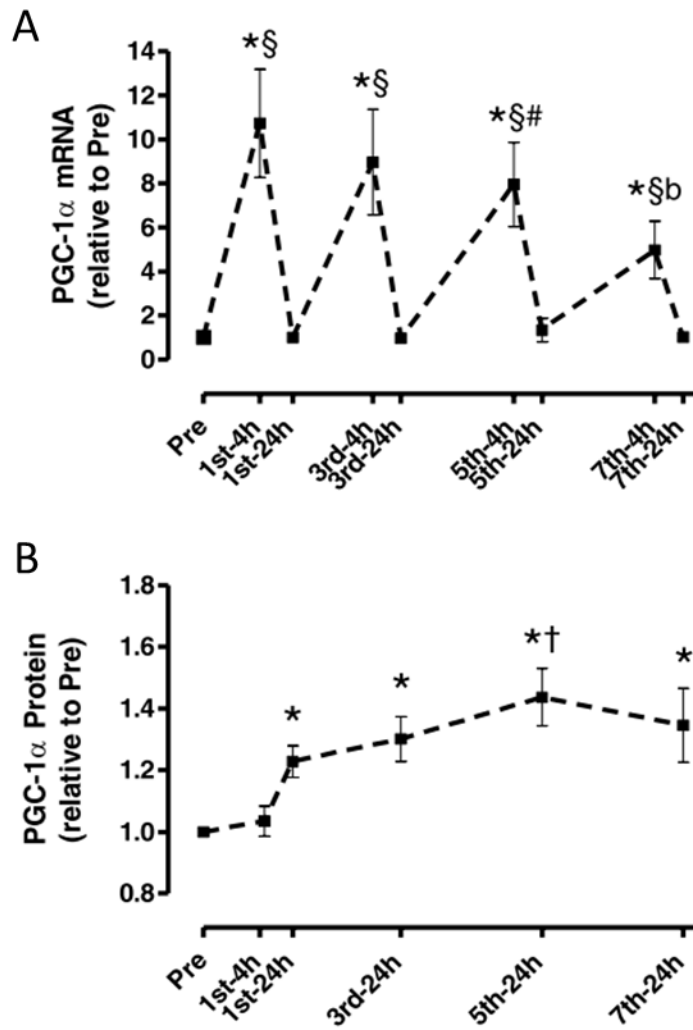
Subsequent studies have also investigated the cellular responses of energy sensitive signaling kinases as well as transcription factors related to mitochondrial biogenesis when an acute bout of exercise was commenced with reduced muscle glycogen concentration as a result of prior exercise/diet interventions. Yeo et al., (2010) investigated the effects on acute signaling responses, in well-trained endurance cyclists, of a single bout of high-intensity interval training (HIT;  $8 \times 5$  min at 85% of  $\text{VO}_{2\text{max}}$ ) commenced with either low or normal muscle glycogen stores. Yeo et al., (2010) reported greater phosphorylation of AMPK when HIT was commenced with reduced compared with normal muscle glycogen availability while

phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) was unchanged for both groups before and after the HIT training sessions. Despite elevated levels of AMPK phosphorylation, no significant differences between trials were observed for several of AMPK's known downstream targets including: nuclear concentrations of histone deacetylase 5 (HDAC5), phosphorylation of cAMP response element binding protein (p-CREB) and phosphorylation of activating transcription factor 2 (p-ATF2).

Other exercise-induced signaling pathways have been shown to be up-regulated after a single bout of exercise commenced with reduced CHO availability. Bartlett et al., (2013) reported that the exercise-induced activation of p53 was glycogen-dependent with a three-fold increase in p53 phosphorylation 3 h post-exercise in a low glycogen state while high CHO availability completely suppressed p53 signaling. The increased p53 response with low CHO availability was also associated with enhanced ACC<sup>Ser79</sup> phosphorylation immediately post exercise through the upstream activation of AMPK but not p38 MAPK phosphorylation.

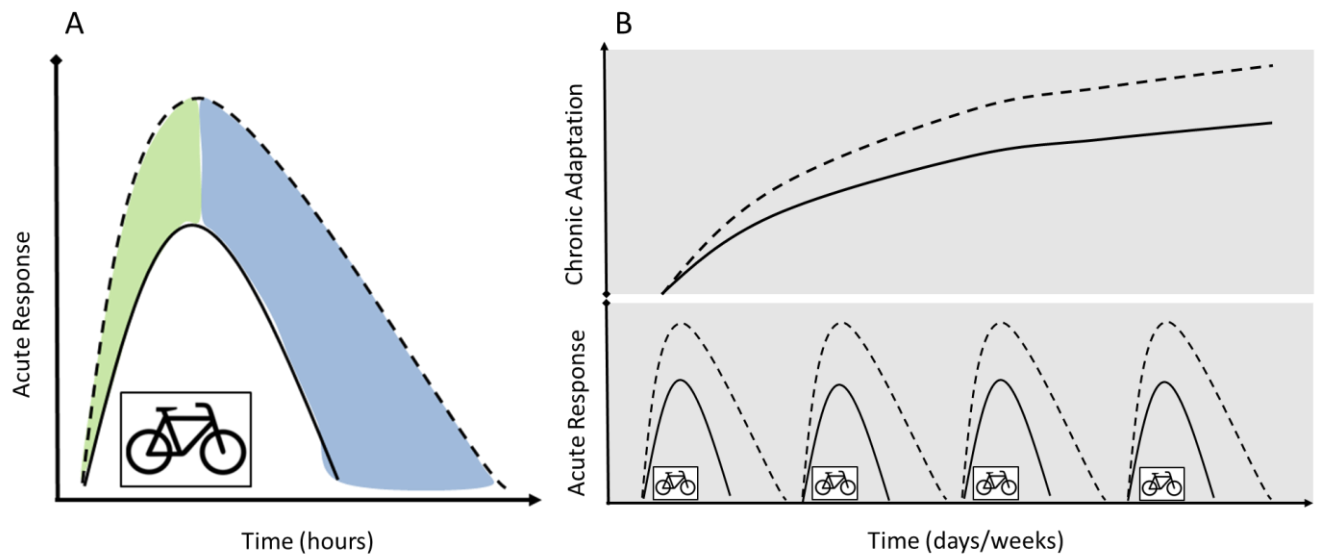
Exercise has been shown to up regulate the activity of an array of genes associated with substrate utilisation and mitochondrial biogenesis (Mahoney et al., 2005, Stepto et al., 2009). It is also clear that selected subsets of genes associated with endurance training adaption are up regulated to a greater extent when exercise is commenced with reduced CHO availability (Churchley et al., 2007, Pilegaard et al., 2002, Pilegaard et al., 2005, Psilander et al., 2012, Steinberg et al., 2006). It is hypothesised that the adaptive increases in transcriptional and metabolic proteins follow the accumulation of transient increases in mRNA after successive exercise sessions. To test this hypothesis Perry et al., (2010) investigated the effects of seven-sessions of high-intensity interval training over a period of two weeks on the time course of responses of mitochondrial biogenesis and selected transcriptional and mitochondrial mRNAs and proteins. The results of Perry et al., (2010) provide mechanistic insight into the time

course of the mitochondrial adaptive response after a single bout of exercise in human skeletal muscle. In particular their results revealed that daily bouts of exercise resulted in transient increases in PGC1 $\alpha$  mRNA, and that these ‘bursts’ of increased mRNA abundance preceded increases in transcriptional and mitochondrial proteins during the training intervention (see Figure 1.2). Over the course of the two week investigation, these transient ‘pulses’ of increased mRNA expression resulted in the accumulation of new muscle proteins, which constitute ‘training adaptation’ and subsequent improvements in metabolic and endurance capacity.



**Figure 1.2** Time-course of exercise induced PGC1α repeated transient bursts and protein accumulation. (A) PGC1α mRNA and (B) the subsequent increase in PGC1α protein content during two weeks of high-intensity exercise in human skeletal muscle. \*Significantly different from Pre; § Significantly different from all 24 h time points; # Significantly different from 1<sup>st</sup> 4 h; b Significantly different from all 4 h time points; †Significantly different from 1<sup>st</sup> 24 h (all  $P < 0.05$ ). (Figure reproduced from Perry 2010)

Collectively the observed ‘pulse’ in acute training responses reported by Perry et al., (2010) and the observed enhanced signaling (Yeo et al., 2010) and gene responses (Pilegaard et al., 2002) when exercise is commenced with reduced muscle glycogen provide important mechanistic insight into skeletal muscle adaptive responses. In particular the reported upregulation of PGC1 $\alpha$  when exercise is commenced with reduced glycogen availability (Psilander et al., 2012, Bartlett et al., 2013) supports the notion that by enhancing the magnitude and duration of each acute ‘pulse’ through exercise nutrient interventions a faster rate of improvement in physiological capacity may be attained. Such a scenario is represented schematically in Figure 1.3.



**Figure 1.3.** Cellular responses of exercise commenced with normal or reduced muscle glycogen content. Acute (A) and chronic repeated (B) exercise and recovery commenced with normal (solid lines) or reduced (dotted line) CHO availability; Amplified acute training response (shaded green) represents exercise commenced with reduced muscle glycogen; Prolonged post-exercise training response (shaded blue) represents acute recovery from exercise where CHO ingestion is withheld and the possible suppression of energy sensitive pathways is delayed. Repeated exposure to enhanced acute training responses may result in a greater rate of accumulation in functional proteins with important roles in endurance-training adaptation.

### *1.2.3 Reduced muscle glycogen availability and training adaptation*

Several studies have investigated specific ‘train-low’ protocols to determine if the augmented acute cellular response when extended into a periodised training programme results in increased functional markers of training adaptation and subsequent performance. In the first study of its kind Hansen et al., (2005) proposed that training commenced with low muscle glycogen availability would promote adaptation to a greater extent than when the same sessions were commenced with normal glycogen concentration. To test this hypothesis Hansen et al., (2005) studied previously untrained male subjects who completed a training programme of single-leg knee extensor exercise 5 d·wk<sup>-1</sup> for 10 wk. Both of the subject’s legs were trained according to a different schedule with the total amount of work undertaken by each leg over the duration of the investigation the same: one leg was trained twice a day, every second day (LOW) with the second session of the day being commenced with lowered muscle glycogen content. Whereas, single daily sessions on the contra-lateral leg allowed adequate time for glycogen re-synthesis between sessions (HIGH). Resting muscle glycogen content before the 10 wk training intervention was similar for both legs but was significantly increased in only the LOW leg post-training. There was a training-induced increase in the maximal mitochondrial enzyme activities of citrate synthase (CS) and  $\beta$ -hydroxyacyl-CoA dehydrogenase ( $\beta$ -HAD) in both legs, but the magnitude of increase was greater in LOW than HIGH. Exercise performance (measured as the time to exhaustion at 90% of post-training maximal power output) was similar for both legs pre training. Noticeably, the magnitude of increase in post-training exercise time to exhaustion was twice as great for LOW as HIGH. The results clearly demonstrated that adaptation was augmented by a lack of substrate (i.e, muscle glycogen) availability, at least for previously untrained subjects undergoing a short-term training intervention. Hansen et al., (2005) coined this novel training approach ‘train-

low, compete-high' and several studies have utilised similar protocols in subsequent investigations.

Using a more 'sport specific' approach, two independent research groups completed investigations using a similar 'train-low' model. Yeo et al., (2008b) and Hulston et al., (2010) investigated the effects of training once daily compared to training twice every second day on skeletal muscle adaptation and performance changes. At a time when glycogen stores were lowered by ~50% through prior exercise the 'train-low' group completed high intensity interval training sessions (HIT) consisting of 8 x 5 min cycling intervals at a self-selected maximal intensity. Both studies reported a reduction in maximal self-selected power output when athletes commenced the intervals with low compared with normal glycogen availability. Yet, despite a lower 'training impulse' Yeo et al., (2008b) reported that resting muscle glycogen concentration, the maximal activities of CS and  $\beta$ HAD and the total protein content of cytochrome c oxidase subunit IV (COXIV) were higher (compared to pre-training values) only in subjects who commenced interval training with low muscle glycogen content. In the study completed by Hulston et al., (2010) similar increases in  $\beta$ HAD were observed, and in addition tracer-derived measures of fat oxidation during sub-maximal cycling were increased after 'train-low'. Specifically, muscle-derived triacylglycerol oxidation increased after training with low glycogen availability. Notably, in the studies of Yeo et al., (2008) as well as Hulston et al., (2010) no differences between groups (HIGH vs LOW) in the degree of improvement (~10%) were observed in pre- and post-intervention performance time trials (~60 min) commenced ~15 min after a pre-load cycling bout (60 min at ~70%  $\text{VO}_{2\text{max}}$ ). Hence, the augmented response to training at the molecular level of the low group did not appear to translate to performance enhancements.



#### *1.2.4 High fat or low glycogen?*

Reviews on the topic of glycogen mediated adaptive responses have clearly highlighted that a range of systemic and intracellular changes also occur in parallel to glycogen depletion during and post exercise (Philp et al., 2012, Hawley and Burke, 2010). Although much evidence has suggested that the reduction in muscle glycogen content augments the acute training response, it is also possible that: i) the contaminant elevation in circulating FFA, ii) increases in sympathetic nervous system activation and subsequent elevation in catecholamines, iii) cellular stress evoked by shifts in intracellular osmotic gradients and/or iv) increased myokine production may also influence the observed superior training response (Philp et al., 2012). Evidence in support of a role for FFA's in the adaptive process come from studies in animals that report chronically elevating FFA concentrations results in increased levels of mitochondrial biogenesis in rat skeletal muscle (Garcia-Roves et al., 2007). Fillmore et al., (2010) observed that a 6 wk high fat diet (chow-fed) in rats elevated PGC1 $\alpha$  and PPAR $\delta$  protein content compared to a control. From this observation Fillmore et al., (2010) suggested that in humans, exercise induced elevations increasing circulating FFA may contribute to exercise training-induced mitochondrial biogenesis and furthermore, it is reasonable to consider that increases in skeletal muscle mitochondrial capacity could be enhanced if training were performed under conditions that further elevated the levels of circulating FFA (Fillmore et al., 2010).

In humans, Tunstall et al., (2007) used acipimox treatment to suppress lipolysis during exercise and subsequent recovery and found that this did not blunt the rise in the mRNA abundance of PDK4 and PGC1 $\alpha$  compared to a placebo. This study concluded that the normal increase in circulating concentrations of FFA during the later stages of exercise and subsequent recovery was not required to induce skeletal muscle mRNA expression of several proteins involved in regulating substrate metabolism (Tunstall et al., 2007). Watt et al.,

(2004) reported that incubating human primary myotubes with epinephrine increased PGC1 $\alpha$  independently of changes in p38 MAPK phosphorylation. This suggests that exercise induced elevations in plasma catecholamine levels have the capacity to directly affect mitochondrial transcription factors and co-activators.

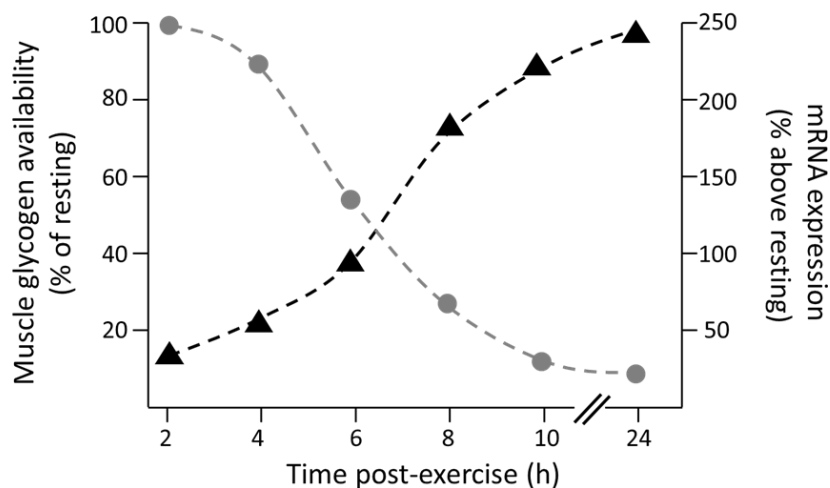
It is clear that exercise evokes a myriad of responses other than solely the depletion of muscle glycogen and these factors have the potential to influence the degree of activation in cellular pathways pertaining to mitochondrial biogenesis and substrate metabolism. Specific exercise nutrient interventions have the capacity to either promote or suppress many of these factors. However, it is currently unclear as to the optimal 'metabolic milieu' for the maximal promotion of specific adaptive responses that result in a shift toward an endurance phenotype.

#### *1.2.5 Effects of carbohydrate re-feeding post exercise*

Current nutritional guidelines advocate the ingestion of a high CHO meal post-exercise to replenish muscle glycogen stores to promote recovery for subsequent exercise (Burke, 1997, Burke et al., 2004). Rapid replenishment would maximise the chance of the athlete commencing subsequent exercise with full glycogen stores. However, while this may facilitate performance during subsequent exercise the restoration of energy stores during recovery via CHO ingestion, may suppress the stimulus for adaptive responses.

It is clear that the regulation of gene expression in skeletal muscle can be altered by dietary interventions (i.e. high fat and low CHO availability), especially glycogen content. It is plausible that by delaying CHO feeding and prolonging the time athletes spend in a low glycogen state may augment the acute training response. However, studies directly investigating the effects of nutritional interventions on acute training responses during

recovery from endurance exercise have gained little attention. This inverse relationship in glycogen content and gene activity has recently been illustrated by Hawley (2013) and is depicted in Figure 1.4. It can be hypothesised that selected nutrient sensitive genes would remain elevated if CHO feeding is delayed post exercise compared to when athletes follow current nutritional guidelines and consume a CHO rich meal early during recovery.

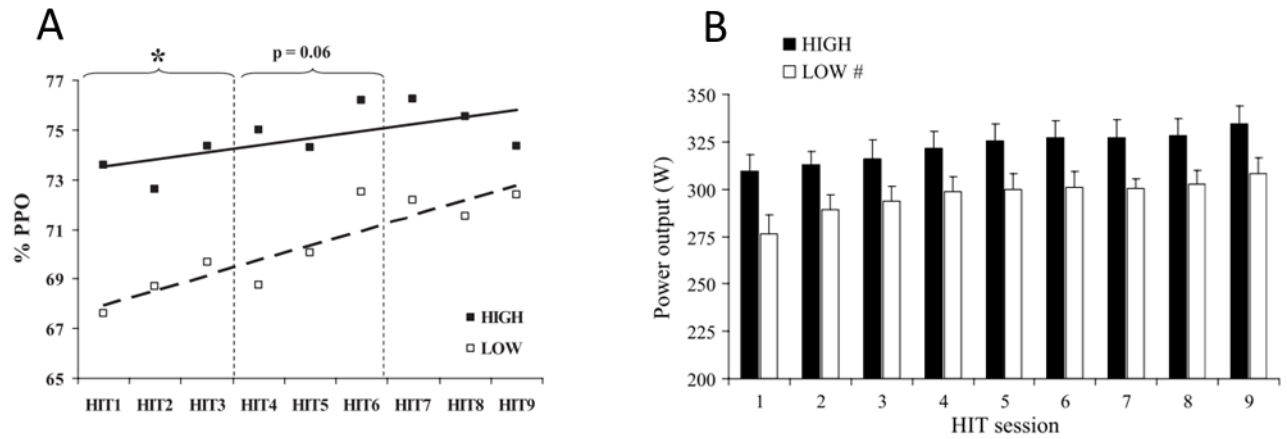


**Figure 1.4.** Hypothetical schematic of the effects of muscle glycogen on the time course of mRNA abundance post exercise. The mRNA expression of ‘exercise-induced metabolic genes’ with putative roles in endurance training adaptation (grey circles) and the restoration of muscle glycogen stores (black triangles) following a single bout of prolonged, glycogen-depleting exercise. The activation of many exercise induced genes peaks in the immediate hours of recovery (1–4 h) and have returned to basal levels within 24 h of the last exercise bout. Low muscle glycogen availability enhances and prolongs the time course of transcriptional activation of many ‘metabolic genes’ in response to exercise, raising the possibility that common signaling pathways sensitive to muscle glycogen availability and/or systematic factors (fatty acid availability and/or hormone levels) may be linked to the transcriptional status of selected genes (Reproduced from Hawley 2013).

To determine the potential influence of substrate availability on the transcriptional regulation of metabolic genes during recovery from exercise Pilegaard (2005) had 9 male subjects complete 75 minutes of cycling exercise at 75%  $\text{VO}_{2\text{max}}$  on two occasions, consuming either a high- or low-CHO diet during the subsequent 24 h of recovery. The main findings from that study were that providing a high CHO diet during recovery suppressed the activation of pyruvate dehydrogenase kinase 4 (PDK4), uncoupling protein 3 (UCP3), lipoprotein lipase (LPL), and carnitine palmitoyltransferase I (CPT1) during the first 5 to 8 hours after exercise, whereas providing a low-CHO diet during recovery elicited a sustained/enhanced increase in activation of these genes throughout recovery (Pilegaard et al., 2005). These observations provide the basis for the development of nutritional guidelines for athletes that focus on enhancing training adaption rather than the traditional guidelines that solely focus on recovery of glycogen stores for subsequent exercise.

#### *1.2.6 Muscle glycogen availability and reduced work capacity*

Commencing exercise with reduced muscle glycogen content diminishes the ability to perform high intensity exercise as well as significantly increasing perceived exertion during submaximal exercise (Wojtaszewski et al., 2003, Rauch et al., 2005). As previously discussed (see Section 1.2.3) the studies of Yeo et al. (2008b) and Hulston et al. (2010) which both incorporated a similar ‘train-low’ approach, showed that maximal self-selected power output was reduced when athletes commenced exercise with low compared with normal glycogen availability. In both studies the group that commenced a high intensity cycling interval training session (8 x 5 min intervals) at their maximal self-selected power output with low muscle glycogen had lower mean power outputs (~8%) compared to the group that commenced the intervals with normal muscle glycogen content (Figure 1.5).



**Figure 1.5** Effects of muscle glycogen on cycling mean power output when high intensity exercise is commenced with HIGH or LOW muscle glycogen. Nine bouts were performed over a 3 wk training intervention. Each high intensity training (HIT) bout consisted of 8 x 5 min cycling intervals at a maximally self-selected intensity; \*# Significantly different between trials. (A; Reproduced from Yeo et al., 2008b); (B; Reproduced from Hulston et al., 2010)

Stepto et al. (2002) reported that after 4 days of a high-fat low CHO diet, (which has been shown to reduce pre-exercise muscle glycogen concentrations to  $\sim 255 \text{ mM} \cdot \text{kg}^{-1}$  dry wt) (Burke et al., 2000) six of seven well-trained cyclists/triathletes were able to complete an identical HIT bout (8 x 5 min) with intensity during the intervals held constant at  $\sim 82\%$  PPO. Taken collectively these results suggest that reduction in self-selected training intensity during HIT in the Yeo et al., and Hulston et al., investigations could be due to a combination of factors and not just a lack of CHO availability. One possibility of course, is the residual fatigue associated with two training sessions undertaken in close proximity.

Yet, despite a reduced maximal self-selected training intensity observed when exercise was commenced with reduced glycogen availability both the acute and chronic training effects appear to be amplified. Consequently, the development of strategies that utilise this 'train-low' approach but avoid the observed reduction in training intensity requires further investigation. It has been proposed that if exercise intensity can be rescued with nutritional/ergogenic interventions when selected sessions are commenced with reduced muscle glycogen the acute signaling response may be even further enhanced (Philp et al., 2011). This premise is based on the notion that a further training stimulus would be gained if the training intensity could be restored. Possible strategies to achieve this include the use of several ergogenic aids to potentially restore training intensity. An alternative strategy to preserve overall training intensity is to complete a high intensity training session first while glycogen availability is not compromised, and then perform a subsequent lower intensity aerobic training bout, in which training intensity would less likely be adversely affected by the reduced glycogen availability, while still preserving the augmented training response.

### *1.2.7 Summary*

Interventions that manipulate CHO availability during and after exercise including the acute recovery period have the capacity to augment several of the acute responses to training and enhance subsequent adaption. Approaches that restore the ‘train-low’ associated reduction in training intensity through the use of ergogenic aids or the programming of training sessions so that high intensity sessions are commenced with high CHO availability are of potential benefit. If training intensity can be ‘rescued’ the magnitude of the acute response may be even further increased. Another possibility is that by restricting CHO during exercise or throughout the acute post-exercise recovery period, signaling events sensitive to CHO availability and the exercise induce metabolic state would remain elevated for longer and thereby prolong the exercise induced stimulus.

## **1.3 Ergogenic Aids**

### **1.3.1 Introduction**

An ergogenic aid is broadly defined as a substance that is work enhancing. The majority of ergogenic aids used to enhance endurance performance fall under three general classifications: i) nutritional, that enhances work capacity through acting as an energy source (e.g. CHO); ii) physiological that are naturally occurring in body, and when supplemented in the diet have an ergogenic effect (e.g. dietary nitrate); and iii) pharmacological interventions that when introduced into the body have specific actions that enhance work capacity (e.g. caffeine). Some ergogenic aids such as caffeine have been the focus of research for decades, whereas more contemporary ergogenic aids such as beetroot juice have less evidence to support their effects. This section of the literature review will focus on three ergogenic aids used in sport today; i) caffeine, ii) CHO mouth rinse and iii) nitrate ( $\text{NO}_3^-$ ) supplementation. Potential mechanisms of action, dose response, modes of ingestion and performance benefits under nutritional conditions typical of elite athletes will be reviewed. The potential use of these ergogenic aids under conditions of either high or low CHO availability will also be discussed.



### ***1.3.2 Caffeine supplementation***

Much of our current understanding of the mechanisms and application behind caffeine's performance enhancing effect has evolved from early studies by the laboratories of Costil, Ivy and Spriet commencing in the mid 1970's. Since these early studies, subsequent research has investigated the optimal dose, timing and implementation of caffeine under a variety performance tasks. It is now well accepted that caffeine improves exercise performance in a range of sporting situations. Caffeine, chemically known as trimethylxanthine, has three metabolites which also contribute to caffeine's physiological effects. i) Paraxanthine is responsible for increases in rates of lipolysis, ii) theobromine is a vasodilator, and iii) theophylline acts as a smooth muscle relaxant and is also known as a chronotrope and inotrope that increases heart rate and force of myocardial contraction (Dews, 1982). Various reviews have detailed caffeine's application and efficacy in sport (Burke 2008, Goldstein et al., 2010). Similarly, the culmination of decades of research has led to the development of a host of guidelines pertaining to withdrawal, dose and form (eg, coffee, anhydrous capsule, energy beverages or chewing gum) of caffeine ingestion with the goal to provide athletes and coaches with the best methods of application.

#### ***1.3.2.1 Caffeine and performance***

Many studies have investigated the dose response characteristics of caffeine that elicit both metabolic and performance changes. Early research used large caffeine doses relative to body mass (BM) (5-13 mg·kg<sup>-1</sup> BM) (Pasman et al., 1995, Graham and Spriet, 1991, Graham and Spriet, 1995, Spriet et al., 1992) compared to more recent studies that are now focusing on smaller doses (1-5 mg·kg<sup>-1</sup> BM) which still appear to exhibit similar metabolic effects, with comparable performance improvements to larger doses of caffeine (McClaran and Wetter,

2007, Jenkins et al., 2008, Desbrow et al., 2009, Desbrow et al., 2012, Ryan et al., 2012, Wiles et al., 2006). Early studies such as that of Pasman et al., (1995) (employing caffeine doses of 5, 9 and 13 mg·kg<sup>-1</sup> BM) required subjects to perform a time to exhaustion cycling task at 80% of maximal power output. All three caffeine treatments significantly delayed fatigue by ~20% compared to placebo with no statistical differences between trials. These results suggest that a moderate (5 mg·kg<sup>-1</sup> BM) dose of caffeine was just as effective in delaying fatigue compared to a high (13 mg·kg<sup>-1</sup> BM) dose. In a study that used running time to fatigue at a comparable intensity (85% VO<sub>2max</sub>) Graham and Spriet (1995) reported similar positive findings with subjects running ~20% longer using a low (3 mg·kg<sup>-1</sup> BM) and moderate (6 mg·kg<sup>-1</sup> BM) dose of caffeine compared to a placebo, but not for 9 mg·kg<sup>-1</sup> BM. The study concluded that the lack of improvement in the 9 mg·kg<sup>-1</sup> BM trial compared to placebo may have been that the high dose of caffeine over stimulated the central nervous system in some subjects which actually resulted in a reduction in performance.

More recently Desbrow et al., (2012) in a study using a more 'race like' performance task (comparative to a time to fatigue test as described previously) in which subjects completed a cycling time trial lasting approximately 1 h. A placebo, 3 or 6 mg·kg<sup>-1</sup> BM of caffeine was administered 90 min prior to the time trial. The results of this study indicated that caffeine was ergogenic to endurance cycling performance, with statistically significant enhancements in time trial times of 4.2% for the lower dose caffeine and 2.9% for higher dose when compared to placebo. These workers concluded that there was no dose response for caffeine on performance and therefore no additional benefit to be gained by ingesting 6 versus 3 mg·kg<sup>-1</sup> BM. The magnitude of the effects demonstrated in this study appear to be consistent with data from previous studies using cycle time trials of 30–60 min duration which have demonstrated performance improvements ranging between 1.8–5.8% (Jenkins et al., 2008, Kovacs et al., 1998).

The effects of 1, 2 and 3 mg·kg<sup>-1</sup> BM of caffeine on cycling performance was investigated by Jenkins et al., (2008) who measured the amount of work completed (kJ·kg<sup>-1</sup> BM) during a 15 min time trial. They reported a 3-4% improvement in work completed in the 2 and 3 mg·kg<sup>-1</sup> BM trials compared to placebo. However, performance after ingestion of 1 mg·kg<sup>-1</sup> BM did not improve performance suggesting that the minimum threshold for performance enhancement is likely to be between 1 and 2 mg·kg<sup>-1</sup> BM.

The efficacy of caffeine under specific conditions of reduced carbohydrate availability has gained little attention. Studies have investigated the effects of caffeine ingestion on cycling time trial performance commenced after a steady state 'pre-load' bout (Cox et al., 2002, Desbrow et al., 2009, Slivka et al., 2008). Such conditions would not only result in reductions in glycogen concentrations but would also be associated with other factors including central and peripheral fatigue. Due to different trial protocols and timing of caffeine administration, clear conclusions regarding the effectiveness of caffeine ingestion after a pre-fatiguing ride are somewhat unclear. A caffeine dose of 6 mg·kg<sup>-1</sup> BM has been shown to enhance time trial performance after pre-fatiguing exercise (Cox et al., 2002) however, lower doses (1.5 and 3 mg·kg<sup>-1</sup> BM) appear to be less effective under these conditions (Desbrow et al., 2009). In addition, it has been reported that caffeine ingestion did not enhance performance during a cycling time trail commenced after 2 days of a reduced calorie diet, resulting in a negative energy balance (Slivka et al., 2008). Further research is required to determine the efficacy of caffeine under conditions of reduced carbohydrate availability to allow the development of clear guidelines that can be employed during training and competition.

It is notable that both the performance enhancing as well as subjective perceptual and physiological effects of caffeine show considerable individual variation. Most frequently it is proposed that differences arise between habitual and non-habitual users (Goldstein et al.,

2010, Tarnopolsky and Cupido, 2000) and that the prior withdrawal period of caffeine ingestion may influence its effectiveness as an ergogenic aid (Irwin et al., 2011, Robertson et al., 1981). However, caffeine has been shown to elicit the same positive response in habitual users regardless of an enforced withdrawal period ranging from 48 h (Van Soeren and Graham, 1998) to 4 days (Irwin et al., 2011). Meanwhile, some individual variation in metabolic effects such as glycogen sparing have been reported by Chesley et al., (1998) indicating that although caffeine ingestion promotes robust performance enhancements the metabolic effects may display individual variation. As such, it is important to consider the use of caffeine on an individual basis, as well as factors such as prior use, withdrawal period, dose and mode of intake.

#### *1.3.2.2 Performance enhancement in cycling time trials*

A summary of studies investigating the effects of different modes of intake, doses and timing of ingestion of caffeine on endurance cycling performance tasks is presented in Table 1.1. Collectively these studies suggest consistent improvement in cycling performance after the ingestion of caffeine in a range of doses from 2-6 mg·kg<sup>-1</sup> BM with performance improvements of up to 6%. No clear dose response relationship appears to be evident with varying individual responses being noted in a number of studies. The evidence for an optimal timing of ingestion as well as form is somewhat unclear. Ingestion ~60 min prior to a performance task in a variety of forms including; anhydrous capsules, caffeine and CHO solutions, coffee and caffeinated gums all appear to elicit similar performance enhancing effects.

**Table 1.1.** Studies investigating the effects of caffeine ingestion on cycling time trial performance.

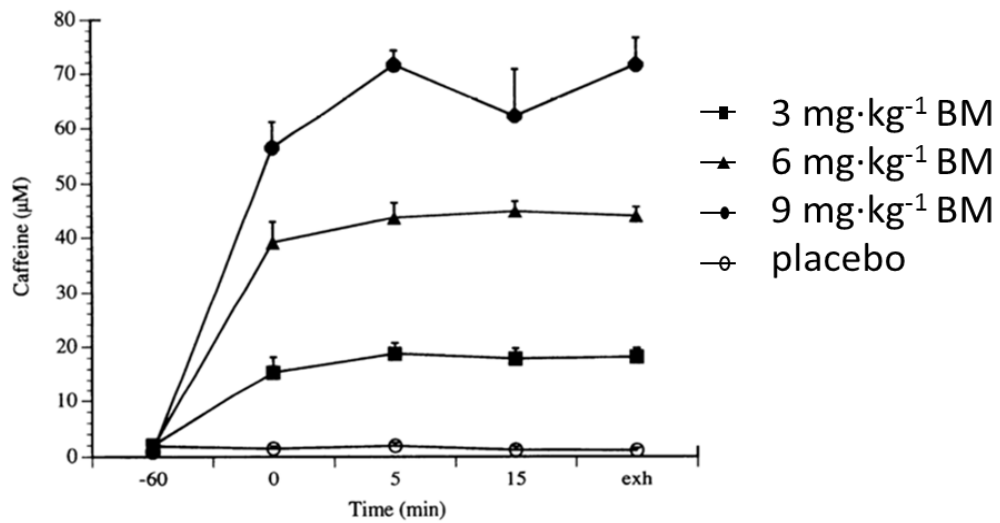
Authors	Year	Performance task	Caffeine form	Caffeine dose	Timing of ingestion	Improvement (p < 0.05)	Effects relative to placebo		
							Time	Work	Mean % improvement
Cox et al.	2002	Cycling; 120 min at 80% VO <sub>2</sub> peak (SS) + 7 kJ·kg <sup>-1</sup> BM TT	Anhydrous capsule or Coke	a) 1 x 6 mg·kg <sup>-1</sup> BM b) 6 x 1 mg·kg <sup>-1</sup> BM c) Coke; 2 x 5 mL·kg <sup>-1</sup> BM (~1.5 mg·kg <sup>-1</sup> BM CAFF)	a) 60min prior to SS b) each 20min in SS c) at 100-120 min SS	Yes	P; 29:18 ± 0:44 a) 28:18 ± 0:40 b) 28:24 ± 0:57 c) 28:24 ± 0:30		a) 3.4% b) 3.1% c) 3.3%
Wiles et al.	2006	Cycling; 1km TT	Anhydrous CAFF dissolved in solution	a) 5 mg·kg <sup>-1</sup> BM b) Control (no placebo or CAFF) c) placebo	60 min prior to TT	Yes	a) 71.1 ± 2.0s b) 73.3 ± 2.7s c) 73.4 ± 2.3s	a) 523 ± 43 W b) 504 ± 38 W c) 505 ± 46 W	a-b) 3.0% a-c) 3.1%
Jenkins et al.	2008	Cycling; 15min 80% VO <sub>2</sub> peak + 15 min TT	Anhydrous capsule	a) 1 mg·kg <sup>-1</sup> BM b) 2 mg·kg <sup>-1</sup> BM c) 3 mg·kg <sup>-1</sup> BM	60 min prior (80min prior to TT)	Yes (2 and 3mg·kg <sup>-1</sup> BM dose only)		P; 2.96 ± 0.16 kJ·kg <sup>-1</sup> BM a) 2.94 ± 0.12 kJ·kg <sup>-1</sup> BM b) 3.08 ± 0.16 kJ·kg <sup>-1</sup> BM c) 3.05 ± 0.17 kJ·kg <sup>-1</sup> BM	b) 3.9% c) 2.9%
McNaughton	2008	Cycling; 60 min TT	CAFF in low kJ flavoured drink	a) 6 mg·kg <sup>-1</sup> BM b) Control (No drink or CAFF) Placebo only drink	60 min prior to TT			P; 26.4 ± 1.5 km a) 28.0 ± 1.3 km b) 26.3 ± 1.5 km	a) 6%
Slivka et al.	2008	Cycling; 120 min at 50% W <sub>max</sub> (SS) + 20km TT; Trials performed in 'live' energy balance with (+CHO) or without (-CHO) CHO	Anhydrous CAFF P) -CAFF/-CHO b) -CAFF/+CHO c) +CAFF/-CHO d) +CAFF/+CHO	Total 800 mg (4 x 200 mg)	+CAFF/-CAFF; 0, 30, 60 and 90 min of SS, +CHO/-CHO; 4 x 500mL (60 g·h <sup>-1</sup> ) at same time as CAFF	No effect of caffeine; Yes -CAFF/+CHO	a) 40.5 ± 7.4 b) 37.2 ± 4.2 c) 38.7 ± 7.1 d) 37.3 ± 4.9		b) 8.1% c) 4.4% d) 7.9%
Desbrow et al.	2009	Cycling; 120 min at 65% PPO (SS) + 7 kJ·kg <sup>-1</sup> BM TT	Anhydrous	a) 1.5 mg·kg <sup>-1</sup> BM b) 3 mg·kg <sup>-1</sup> BM	60 min prior to SS (180min prior to TT)	No	P; 30:25 ± 3:10 a) 30:42 ± 3:41 b) 29:51 ± 3:38		b) -0.6% c) 2.0%

Irwin et al.	2011	Cycling; TT equivalent work to 60min at 75% PPO; (mean work 1040 ± 74 kJ)	Anhydrous capsule	a) 4 days placebo + pre-placebo b) 4days placebo + pre-3 mg·kg <sup>-1</sup> BM CAFF c) 4 days CAFF(2 x 1.5 mg·kg <sup>-1</sup> BM·day <sup>-1</sup> ) + pre-placebo d) 4 days CAFF(2 x 1.5 mg·kg <sup>-1</sup> BM·day <sup>-1</sup> ) + pre-3 mg·kg <sup>-1</sup> BM CAFF	90 min prior to TT	Yes; Effect in pre-CAFF trials	a) 59:53 ± 4:54 b) 58:04 ± 4:14 c) 59:46 ± 4:13 d) 57:39 ± 4:54	a) 291 ± 40 W b) 301 ± 38 W c) 293 ± 37 W d) 305 ± 42 W	a-d) 4.8% <sup>a</sup> c-d) 4.1% <sup>a</sup> a-b) 3.4% <sup>a</sup> b-c) 2.7% <sup>a</sup>
Desbrow et al.	2012	Cycling; TT equivalent work to 60min at 75% PPO		a) 3 mg·kg <sup>-1</sup> BM b) 6 mg·kg <sup>-1</sup> BM	90 min prior to TT	Yes; In 3 mg·kg <sup>-1</sup> BM (a) only	P; 3902 ± 340s a) 3738 ± 286s b) 3791 ± 281s		a) 4.2% b) 2.8%
Ryan et al.	2013	Cycling; 15min 75 VO <sub>2peak</sub> + 7 kJ·kg <sup>-1</sup> BM TT	Caffeine Gum	300 mg	a) 120 min prior to TT b) 60min prior to TT c) 5min prior to TT	'es; In 5 min (c) prior only'	P; 40.7 ± 1.2 a) 42.6 ± 2.2 b) 41.8 ± 2.6 c) 38.7 ± 1.2		a) +4.6% b) +2.7% c) 4.9%
Skinner et al.	2013	Cycling; 40km Cycling	Anhydrous capsule	6 mg·kg <sup>-1</sup> BM	a) 60 min prior to TT b) Ingestion timed to coincide peak plasma CAFF with start of TT	Yes; Improvement in 1 h (a) only compared to placebo	P; 3546 ± 122s a) 3457 ± 97.2s b) 3508 ± 148s		a) 2.0% b) 1.1%
Hodgson et al.	2013	Cycling; 30min at 50% Wmax SS + ~ 650 kJ TT	(600 mL volume) a) Anytdrous in water b) coffee c) decaffeinated coffee d) placebo in water	5 mg·kg <sup>-1</sup> BM	90 min prior to TT	Yes: both CAFF tirials (a and b)	a) 38.5 ± 1.5 b) 38.27 ± 1.8 c) 40.31 ± 1.22 d) 40.23 ± 1.98	a) 294 ± 21 b) 291 ± 22 c) 276 ± 23 d) 277 ± 14	a-d) 6.1% <sup>a</sup> b-d) 5.0% <sup>a</sup> a-c) 6.5% <sup>a</sup> b-c) 5.4 % <sup>a</sup>

<sup>a</sup> - difference in mean power output; P Placebo; SS - Steady state exercise, TT- time trial; PPO - peak power output; W<sub>max</sub> - maximal power output; CAFF - caffeine; BM - body mass; W - watts; CHO - carbohydrate

#### *1.3.2.3 Plasma concentrations and timing of caffeine ingestion*

Caffeine is rapidly absorbed into the blood stream with peak concentrations occurring approximately 30-60 min post ingestion (Magkos and Kavouras, 2005, Mumford et al., 1996). The majority of current studies investigating the effects of caffeine on exercise performance use a single dose administered ~60 min prior to commencement of exercise. Peak concentrations appear to remain for 3-4 h while the half-life of caffeine in the blood is in the range of 4-6 h however, considerable variance between individuals is observed (Graham, 2001). Post ingestion plasma caffeine concentrations appear to peak in a clear linear dose response manner (see Figure 1.6) with peak concentrations in the range of ~20  $\mu\text{M}$  to ~80  $\mu\text{M}$  for 3  $\text{mg}\cdot\text{kg}^{-1}$  BM and 9  $\text{mg}\cdot\text{kg}^{-1}$  BM doses respectively (Graham and Spriet, 1995, Desbrow et al., 2012) with the use of caffeinated gums yielding similar peak concentrations (Ryan et al., 2013)

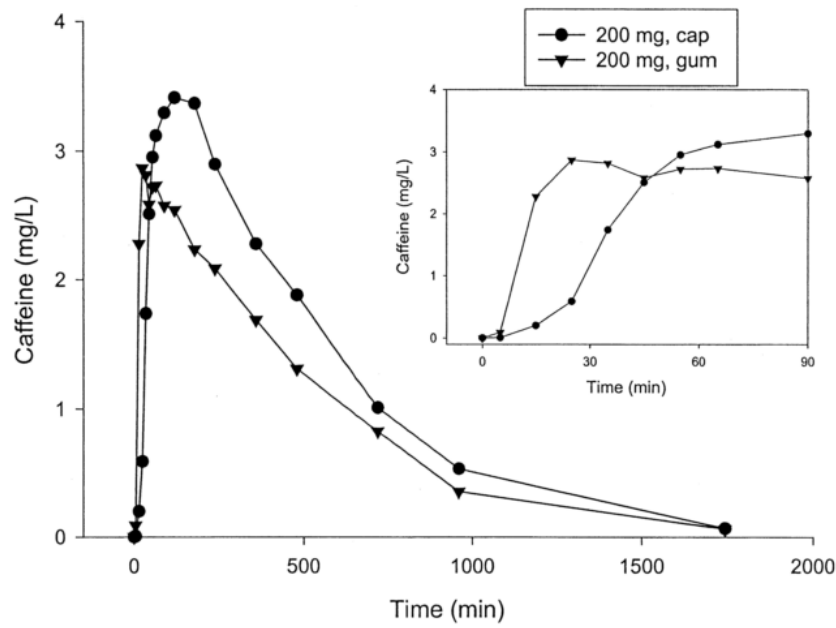


**Figure 1.6.** Plasma caffeine concentration after ingestion of anhydrous caffeine 60 min prior to exercise. A linear dose response is evident with peak concentrations occurring with ~ 60-75 min for all dose concentrations (Figure reproduced from Graham and Spriet 1995)



More recently the use of caffeinated gums as a delivery mode for caffeine administration has become popular in place of the more traditional anhydrous capsule form (Kamimori et al., 2002, Paton et al., 2010, Ryan et al., 2013, Ryan et al., 2012). The rates of absorption of caffeine using a caffeinated gum have been shown to be significantly faster than ingested capsules (Kamimori et al., 2002, Ryan et al., 2013). In comparing the absorption rate of different concentrations of caffeinated gums and capsules Kamimori et al., (2002) concluded that the rate of drug absorption from the gum was significantly faster and may indicate absorption via the buccal mucosa in the mouth (Figure 1.7). In addition, for a dose of 100 and 200 mg, the gum and capsule formulations administered in the Kamimori et al., (2002) investigation induced comparable concentrations of caffeine to the systemic circulation. These findings suggest that there may be an earlier onset of the pharmacological effects of caffeine delivered as a gum formulation.

Skinner et al., (2013) investigated the effects of coinciding the onset of endurance exercise with an individual's peak serum caffeine concentrations compared to the contemporary 1 h prior to exercise guideline. Skinnier et al., (2013) reported that the time to mean peak serum caffeine concentration was 120 min. Coinciding peak serum caffeine levels with the commencement of a 40 km time trial did not enhance performance compared to placebo, whereas when caffeine was ingested 1 h prior, the time to complete the trial was significantly faster than placebo.



**Figure 1.7.** Caffeine absorption profile after ingestion of caffeine gum or anhydrous capsule following a 200 mg dose. Inset shows acute time course of the same ingestion up to 90 min after administration. The time to peak ingestion of caffeinated gum (~25 min) was faster than that for capsule (~90 min) while peak plasma concentrations were marginally higher for capsule. (Figure reproduced from Kamimori,. 2002)

#### *1.3.2.4 Mechanisms of action of caffeine*

There are several proposed mechanisms for the ergogenic effects of caffeine on exercise performance and these have been extensively reviewed (Tarnopolsky, 1994, Meeusen et al., 2013, Spriet, 1995, Tarnopolsky 2008). The most widely reported ergogenic mechanisms, although largely interrelated can be categorised into three main themes: i) metabolic effects, ii) direct effects on skeletal muscle and iii) centrally mediated effects (see Figure 1.8). Many of the effects of caffeine are believed to be related to caffeine's competition with adenosine and its antagonistic effects at adenosine receptors (Daly et al., 1983). The wide abundance of adenosine receptor isoforms, mainly at A<sub>1</sub> and A<sub>2</sub> receptor sites allows this caffeine/adenosine receptor affinity to elicit its effects in a wide range of biological tissues. Therefore, the effects of caffeine are most probably multifaceted with effects occurring on several systems that, when combined, result in improved performance.

Early research proposed that the ergogenic effects of caffeine ingestion may have been linked to metabolic perturbations in substrate utilisation (Graham et al., 2008, Graham and Spriet, 1991, Laurent et al., 2000). Studies observed that caffeine increased the level of intramuscular triglyceride catabolism and reduce muscle glycogenolysis (Costill et al., 1978, Ivy et al., 1979) through mechanisms believed to involve a caffeine mediated suppression of glycogen phosphorylase activity (Chesley et al., 1998). It has also been observed that systemically, through the stimulation of  $\beta$ -adrenergic pathways, caffeine also increase circulating catecholamine concentrations which in turn increases levels of circulating non-esterified fatty acids (NEFA) (Graham and Spriet, 1995). Both this intramuscular and systemic shift toward fat oxidation has been suggested to play a role in the proposed glycogen 'sparing' effect (Costill et al., 1978, Ivy et al., 1979). However, more recent evidence suggests that caffeine in a dose as high as 6 mg·kg<sup>-1</sup> does not elicit this glycogen sparing effect (Graham et al., 2000, Laurent et al., 2000). Although caffeine appears to

influence lipid turnover and oxidation (Acheson et al., 2004) it is still unclear if this is the primary mechanism by which caffeine ingestion enhances prolonged aerobic exercise capacity.

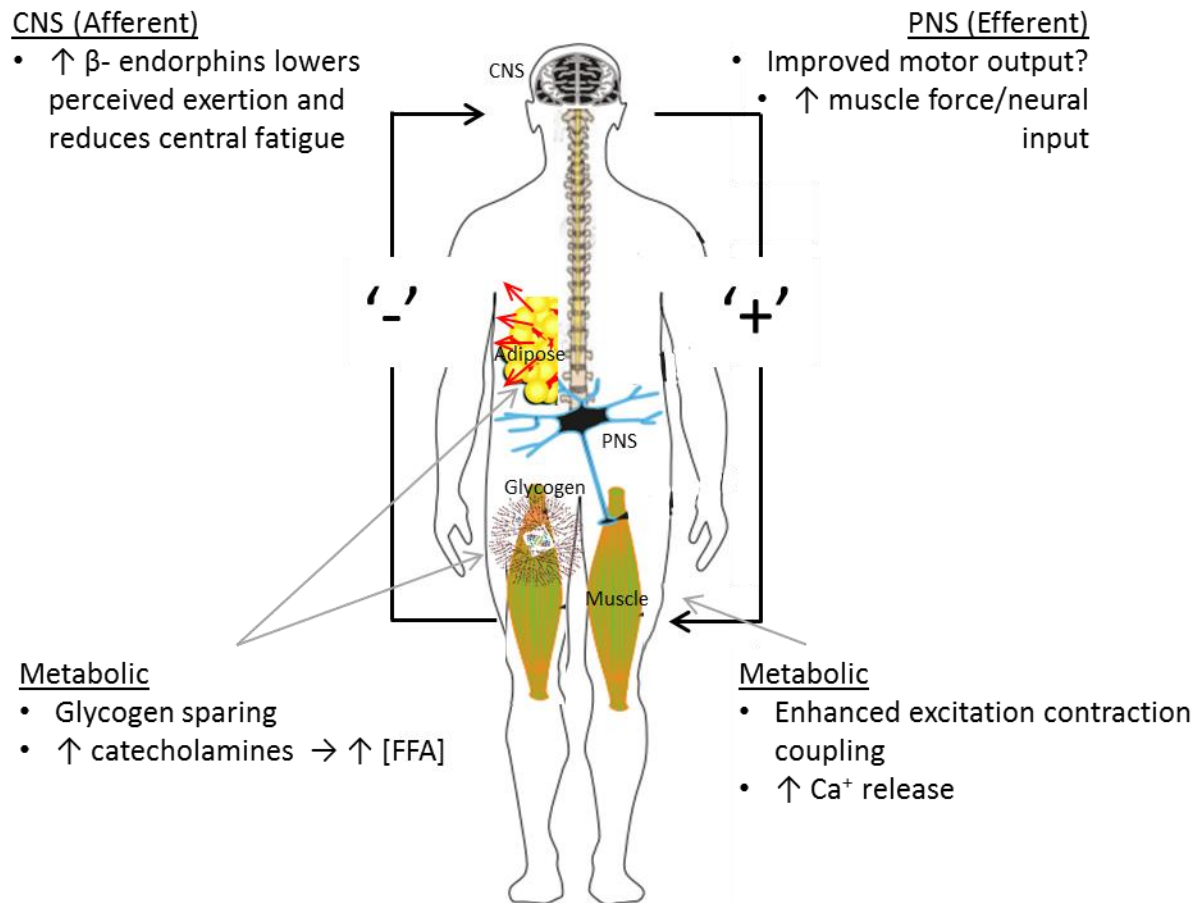
The potential for an ergogenic effect of caffeine on skeletal muscle was first demonstrated when caffeine at physiological doses was shown to directly affect the contractile properties of skeletal muscle undergoing electrical stimulation (Lopes et al., 1983), although, it is less clear if caffeine has the same effect during voluntary maximal contraction (Greer et al., 2006, Kalmar and Cafarelli, 1999). This direct effect of caffeine on contractile force is thought to occur through enhanced excitation contraction coupling (Lindinger et al., 1996). Lindinger et al., (1993) reported that caffeine reduced muscle fatigue through the maintenance of electrolyte homeostasis. In that study it was reported that the ingestion of caffeine prior to exercise aided in the regulation of plasma and intracellular  $[K^+]$  by stimulating  $Na^+/K^+$  pump activity in contracting and inactive skeletal muscle. Caffeine has also been shown to cause a greater mobilisation of calcium from the sarcoplasmic reticulum (Rousseau et al., 1988). This combined with the reported potential of caffeine to lower the mechanical threshold (i.e., the membrane potential at which contraction is induced) (Magkos and Kavouras, 2005) also potentially aids in attenuating muscle fatigue.

Despite a potential direct effect of caffeine on skeletal muscle, the majority of recent studies implementing caffeine as an ergogenic aid ascribe a centrally mediated effect to explain performance improvements (Davis et al., 2003). Many laboratories that once reported a glycogen sparing effect have now recognised the centrally mediated mechanism (Graham et al., 2008, Graham et al., 2000). Caffeine's effects on the central nervous system (CNS) is proposed to occur through the antagonism of the adenosine receptors, influencing the dopaminergic and other neurotransmitter systems (Meeusen et al., 2013). Some of the most

convincing evidence to elucidate a centrally mediated effect of caffeine comes from Davis et al., (2003) who reported that rats who received an intracerebroventricular injection of caffeine ran for 60% longer on a treadmill compared to when a vehicle injection was administered. Because caffeine easily crosses the blood brain barrier, these results would also suggest that orally ingested caffeine has the ability to directly affect the CNS.

The centrally mediated effects of caffeine appear to result in the inhibition of afferent neural pathways resulting in a reduction in perceived exertion (Doherty and Smith, 2005, Demura et al., 2007, Green et al., 2007, Backhouse et al., 2011) and reduced sensations of fatigue and pain (Motl et al., 2003). The observed reduction in perceived exertion after caffeine ingestion is widely reported (Alves et al., 1995, Backhouse et al., 2011, Demura et al., 2007, Doherty and Smith, 2005, Green et al., 2007, Hudson et al., 2008) with significant reductions in perception of effort predominantly apparent during submaximal exercise (Doherty and Smith, 2005). Mechanisms explaining these observations appear to be linked to an increase in circulating  $\beta$ -endorphin which are well known to enhance exercise performance through the ability to decrease pain perception and promote euphoria (Harber and Sutton, 1984). Laurent et al., (2000) reported findings that suggest that caffeine lowers the threshold for exercise-induced  $\beta$ -endorphin and cortisol release, which may contribute to the reported benefits of caffeine on exercise endurance.

Collectively, current evidence suggests that the effects of caffeine are multifactorial with the most pronounced effects of caffeine on performance acting via mechanisms occurring within the CNS. The resulting neuroendocrine responses have the capacity to possibly augment motor output and fatigue perception resulting in greater exercise capacity during submaximal and maximal exercise.



**Figure 1.8.** Known effects of caffeine on body systems that may result in enhanced exercise performance. The reduction of performance inhibiting feedback occurs via afferent mechanisms of reduced pain perception and exertion. Efferent signals are augmented through centrally mediated mechanisms of motivation and peripheral motor unit activation potentially increasing contraction force. Intramuscular and systemic factors reduce glycolysis and increase lipolysis resulting in a glycogen sparing effect. Excitation contraction coupling including increased  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum enhances muscular contractile force. CNS, central nervous system; PNS, peripheral nervous system; FFA, free fatty acids. (Figure adapted from Tarnopolsky 2008)

#### *1.3.2.5 Source of Caffeine*

Various forms of caffeine delivery have been explored. This includes, but is not limited to, anhydrous caffeine capsules (Desbrow et al., 2012, McNaughton et al., 2008, Jenkins et al., 2008, Backhouse et al., 2011, Simmonds et al., 2010), coffee (Hodgson et al., 2013, Demura et al., 2007), sports drinks (Cureton et al., 2007, Del Coso et al., 2012, Duncan and Hankey, 2013, Gwacham and Wagner, 2012), chocolate (Mumford et al., 1996), chewing gum (Kamimori et al., 2002, Paton et al., 2010, Ryan et al., 2013, Ryan et al., 2012) and liquid mouth rinse (Doering et al., 2014, Beaven et al., 2013).

Most commonly studied within the literature is the use of anhydrous caffeine in an opaque capsule. This form allows a controlled dose of caffeine to be administered either in absolute (mg) or relative ( $\text{mg}\cdot\text{kg}^{-1}$  BM) concentrations. This method also allows a placebo to be easily disguised through the use of an identical capsule containing a non-ergogenic substance during ‘blinded’ trial protocols. The use of caffeinated gums has become increasingly popular in the past decade (Ryan et al., 2013, Ryan et al., 2012, Paton et al., 2010, Kamimori et al., 2002) in part for the observed increased rates of absorption compared to traditional forms of caffeine administration (described previously in Section 1.3.2.3). As well as rapid absorption through the buccal mucosa of the oral cavity as seen in the use of caffeinated gums it has also been proposed that when a caffeine solution is rinsed in the mouth there may be a possible near immediate ergogenic effect despite no detectable increase in circulating caffeine concentrations (Beaven et al., 2013). Although, subsequent studies using a caffeine mouth rise protocol did not appear to improve repeated running sprint (Doering et al., 2013) or cycling (Doering et al., 2014) performance.

Coffee is a common source of dietary caffeine (Burke 2008) however, its use in research is less common despite it being reported as the most common source of caffeine consumed by

athletes (Desbrow and Leveritt, 2006). The ergogenicity of coffee compared to anhydrous caffeine has been investigated. Graham et al., (1998) reported that running time to exhaustion at 85%  $\text{VO}_{2\text{max}}$  was improved after 4.5  $\text{mg}\cdot\text{kg}^{-1}$  BM of anhydrous caffeine prior to exercise compared to placebo, whereas, no improvement was observed after the ingestion of coffee containing an equivalent amount of caffeine or with the addition of 4.5  $\text{mg}\cdot\text{kg}^{-1}$  BM of anhydrous caffeine to decaffeinated coffee. There were no differences in plasma caffeine or methylxanthine concentrations for any of the trials that contained caffeine to possibly explain the differences in performance. Graham et al., (1998) proposed that other organic compounds in the coffee may have antagonised the physiological responses of caffeine. More recently the performance effects of caffeine compared to coffee have been investigated by Hodgson et al., (2013). This study found no significant difference in performance enhancement in a cycling time trial after the ingestion of either 5  $\text{mg}\cdot\text{kg}^{-1}$  BM anhydrous caffeine dissolved in 600 mL of water or the equivalent dose of instant coffee in the same volume. On average both trials containing caffeine improved power output compared to placebo and decaffeinated coffee trials by ~5% suggesting that caffeine in the form of coffee can improve performance during a cycling time trial (Hodgson et al., 2013).

#### *1.3.2.6 Summary*

Caffeine has proven to be a reliable ergogenic aid in a wide range of performance tasks. Its benefit in improving cycling power output is in the range of 3-6 % with a resulting reduction in time to complete a set amount of work in the range of 1-6%. Evidence suggests that a dose of 2-6  $\text{mg}\cdot\text{kg}^{-1}$  BM of caffeine ingested 60-90min prior to high intensity exercise is adequate to promote maximal performance enhancements in most individuals with these ergogenic effects of caffeine likely to be centrally-mediated. Caffeinated gums are becoming more



popular with an increased rate of absorption compared to ingested capsules as well as a possible direct effect on receptors in the oral cavity that potentiate rapid ergogenic effects. Evidence also suggests that caffeine can improve performance in states of reduced CHO availability (time trials commenced after pre-depleting exercise) as well as under conditions where exercise is commenced in a rested state with the provision of CHO prior to and during exercise. However, no specific studies have investigated the efficacy of caffeine under conditions of normal or reduced CHO availability.

### ***1.3.3 Carbohydrate mouth rinse***

Carbohydrate ingestion during prolonged exercise improves performance via the maintenance of euglycemia and rates of muscle CHO oxidation (Coggan and Coyle, 1987, Coyle et al., 1986). It is only in the last decade that research has begun to suggest that the benefits of CHO feeding during exercise are not confined purely to a metabolic role but may also be due to a positive afferent signal that improves motor output (i.e. a centrally mediated effect).

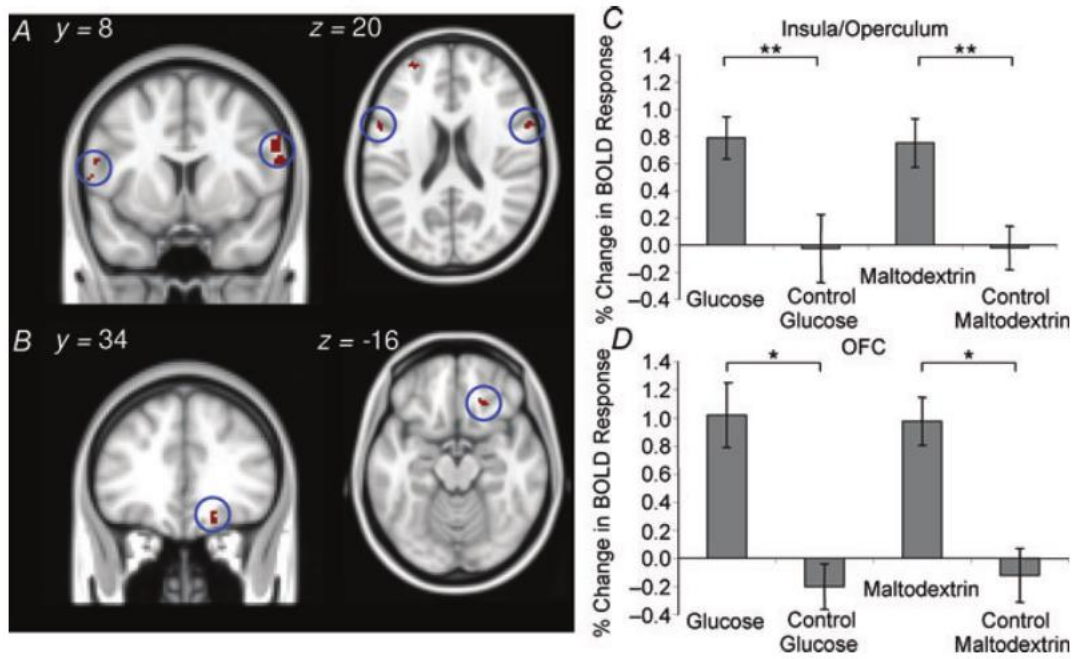
#### ***1.3.3.1 Oral carbohydrate sensing***

The notion of oral CHO sensing and its possible ergogenic role was first raised in a study by Jeukendrup et al., (1997). In that study a 40 km cycling time trial was performed with or without the ingestion of a CHO electrolyte solution. Jeukendrup et al., (1997) reported that CHO feeding during the trial reduced the time to complete the task by 2.3%. This observation prompted the search for an alternate mechanism of action for ingested CHO under the presumption that it afforded no metabolic benefit. The possible role of receptors in the oral cavity that are sensitive to the energy in CHO was later proposed by Carter et al., (2004). To test this hypothesis Carter et al., (2004) had subjects perform a 40 km time trial with a glucose or a placebo infusion. Despite higher blood glucose concentrations and rates of glucose uptake into skeletal muscle with the glucose infusion, performance was not different between trials. This study shows that when by-passing the oral cavity and digestive tract, greater availability of exogenous CHO does not improve high intensity cycling performance lasting ~1 h, thereby highlighting the lack of metabolic effect.

In the first direct evidence of the ergogenic properties of a CHO mouth rinse Carter et al., (2004) investigated the effects of a CHO mouth rinse on 1 h cycling time trial performance. These workers examined whether the provision of a mouth rinse containing 25 mL of a 6.4% maltodextrin (a non-sweet tasting CHO) solution orally administered and then expelled at regular intervals throughout exercise would enhance ~1 h cycle time trial performance compared to a non-CHO matched placebo. The maltodextrin mouth rinse improved performance on average by 2.9% over the placebo trial and it was concluded that the mechanism behind this performance increase may have occurred due to CHO receptors in the oral cavity that modulate central pathways associated with motivation.

#### *1.3.3.2 Mechanisms of action*

To confirm the presence of a ‘central reward mechanism’ induced by the mouth rinse procedure Chambers et al., (2009) used a similar protocol to that employed by Carter et al., (2004) with the addition of investigating possible differences in effect between a sweet (glucose) and non-sweet (maltodextrin) tasting CHO. Chambers and colleagues reported a significant reduction in time to complete the trial under the mouth rinse conditions (Chambers et al., 2009). The novel finding of their study, however, was that using functional magnetic resonance imaging they showed that oral exposure to both glucose and maltodextrin activated reward-related brain regions whereas the placebo (saccharin) did not (see Figure 1.9). These findings help consolidate the hypothesis that there is a central effect of orally ingesting a CHO solution and that even when adequate endogenous CHO stores are present a CHO mouth rinse can enhance performance.



**Figure 1.9.** The activation of brain regions after the ingestion of glucose or maltodextrin.(A) Insula/frontal operculum; (B) Medial orbitofrontal cortex. (C) and (D) average change in response for glucose and maltodextrin and their respective control solutions. This highlights the central mechanism thought responsible for oral CHO sensing is mediated by the caloric value of the solution as both glucose and maltodextrin contain D-glucose units however maltodextrin is a non-sweet tasting CHO. (C and D) \* Significant difference between solutions ( $P < 0.05$ ); \*\* ( $P < 0.001$ ) (Reproduced from Chambers et al., 2009)

#### *1.3.3.3 Carbohydrate mouth rinse and performance*

Since the original work by Carter et al., (2004) a CHO mouth rinse has received growing scientific attention. A number of studies have now investigated the effects of a CHO mouth rinse during a variety of endurance performance tasks under varying nutritional conditions (Carter et al., 2004, Whitham and McKinney, 2007, Rollo et al., 2008, Beelen et al., 2009, Chambers et al., 2009, Pottier et al., 2010, Rollo et al., 2010, Fares and Kayser, 2011, Rollo et al., 2011, Gam et al., 2013). The main findings of studies investigating the effects of a CHO mouth rinse on endurance performance are presented in Table 1.2. The evidence suggests that a CHO mouth rinse increases performance during simulated running and cycling time trials by ~3% with improvements in time to fatigue during moderate intensity submaximal exercise in the range of 3-11%

Despite evidence for an ergogenic effect of orally rinsing a CHO solution, several studies have failed to observe performance improvements (Beelen et al., 2009, Whitham and McKinney, 2007, Rollo et al., 2011). Whitham and McKinney (2007) reported no enhancement in performance between a CHO or placebo mouth rinse in a 45 min running time trial protocol. Similarly, Beelen et al., (2009) reported no difference in mean power output or time to complete a ~1 h cycling time trial utilising a CHO mouth rinse or placebo when performed 2 h after a high CHO meal. Such evidence may suggest that pre-exercise nutritional status may influence the ergogenic effect of a CHO mouth rinse.

**Table 1.2.** Summary of time trial or time to fatigue performance results from studies using a carbohydrate mouth rinse.

Authors	Year	Performance task	Rinse duration	Rinse Timing	Timing of last meal	Effect of rinse relative to placebo			
						Improvement (p < 0.05)	Time	Power	Distance
Carter et al.	2004	~ 1h cycling TT		12.5% total time (7.5min)	4 h	Yes	-2.9%	+2.8%	
Pottier et al.	2010	~ 1h cycling TT	5 sec	12.5% total time (7.5min)	at least 3 h	Yes	-3.7%		
Rollo et al.	2008	30min treadmill running	5 sec			Yes			+2%
Rollo et al.	2011	1 h treadmill running	5 sec	15 min	Over night fast	Yes			+2%
Chambers et al.	2009	~ 1h cycling TT	10 sec	12.5% total time (7.5min)	Minimum 6 h	Yes	-1.9%		
Chambers et al.	2009	~ 1h cycling TT	10 sec	12.5% total time (7.5min)	Minimum 6 h	Yes	-3.1%		
Beelen et al.	2009	~ 1h cycling TT		12.5% total time (7.5min)	2 h	No	+0.91%	-0.37%	
Witham et al.	2007	~ 1h running	5 sec	6 min	4 h	No	-0.5%		+25%
Gam et al.	2013	~ 1h cycling TT	5 sec	12.5% total time (7.5min)					
Fares et al.	2011	Cycling time to exhaustion	5-10sec	5 min	Over night fast	Yes	+11.6%		
		at 60% Wmax	5-10sec	5min	3 h	Yes	+3.5%		

#### *1.3.3.4 Carbohydrate mouth rinse and prandial state*

One possibility for the inconsistencies observed in the effects of a CHO mouth rinse could be the prandial state in which a performance task is commenced. There is a trend for studies that have commenced a performance trial in a post-prandial state to show either a reduced (Fares and Kayser, 2011) or no effect (Whitham and McKinney, 2007, Beelen et al., 2009) of a CHO mouth rinse.

To investigate the interaction of pre-exercise feeding and a CHO mouth rinse on performance Beelen et al., (2009) administered a meal containing ~2.5 g/kg BM of CHO ~2 h prior to a ~1 h cycle time trial. Beelen et al., (2009), reported no significant difference in the time to complete the experimental trial between a placebo mouth rinse or CHO mouth rinse. In a later study Fares and Kayser (2011) also showed a diminished response of a CHO mouth rinse when a cycling time to exhaustion task was commenced in a fed state (3.5% improvement) compared to an overnight fasted state (11.6% improvement). Collectively these findings suggest that the central mechanism responsible for the performance enhancement seen in a fasted state when a CHO mouth rinse is administered is somewhat diminished or non-existent when a high CHO meal is consumed 2-3 h prior to exercise. However, there are currently no investigations that have compared the effects on performance in both a fed and fasted state with both a CHO mouth rinse as well as placebo trials.

#### *1.3.3.5 Summary*

The results of the studies described in this literature review suggest that a CHO mouth rinse has the capacity to improve high intensity endurance performance. In time trial tasks, improvements in time have been observed in the range of 1-5%. The effectiveness of a CHO

mouth rinse appears to be influenced by prandial state where ingestion of meal 2-3 h prior to exercise reduces the effectiveness of the rinse procedure. Future work should aim to determine the practical use of a CHO mouth rinse (i.e., during nutritional practices typical of athletes during competition). Under laboratory conditions the mouth rinse procedure has been performed with a rinse then 'spit' protocol however, under practical conditions there is no evidence to suggest that rinsing then swallowing the solution has any detrimental effect on performance. The application of current knowledge to athletes competing in high intensity endurance events has yet to be extended to a uniform set of guidelines. Due to individual preferences of pre-event feeding or differences in gastrointestinal tolerance the findings of current research may be applied in various ways. Athletes who are susceptible to suffering gastrointestinal upset as a result of performing exercise after a substantial meal or from consuming exogenous CHO during activity may gain benefit from a CHO mouth rinse during an event.



### **1.3.4 Nitrate supplementation**

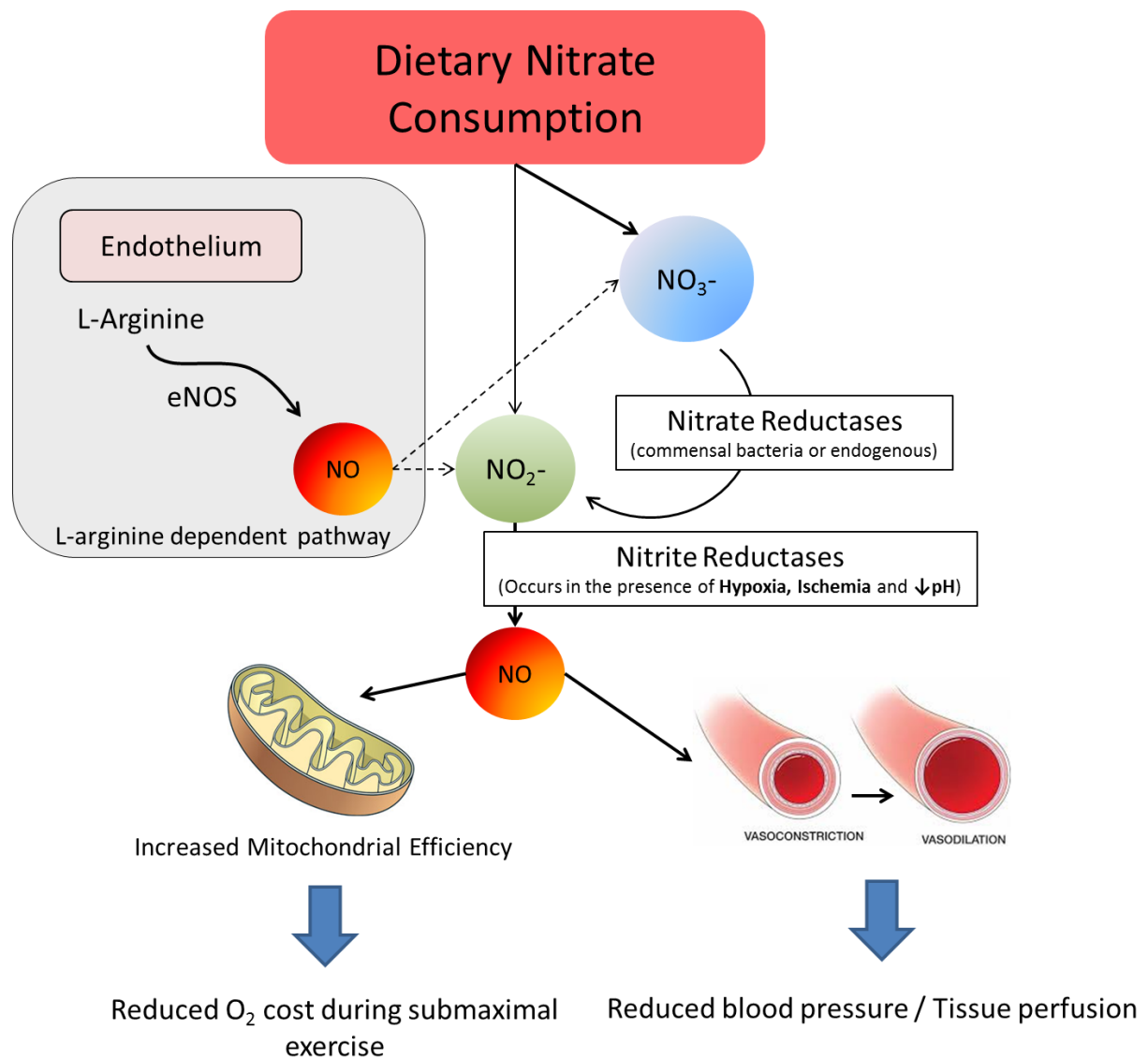
In recent times much interest has arisen regarding the presence of nitrates in the diet and their potential therapeutic and ergogenic properties (Hoon et al., 2013, Jones et al., 2011). Beetroot is a natural and readily available vegetable with a high  $\text{NO}_3^-$  concentration. The use of specific products, including beetroot juice concentrates, which purportedly enhance sports performance, have become increasingly popular. Investigation into the effectiveness of beetroot juice as an ergogenic aid has received much attention. While clear guidelines for its ergogenic use have yet to be developed it does appear to offer a genuine ergogenic effect under certain experimental conditions. Factors such as timing and dosing protocols, intensity of exercise tasks as well as training history of an athlete all appear to influence the effectiveness of beetroot juice supplementation and are now the focus of current and future investigations.

#### **1.3.4.1 Mechanisms of action of nitrates**

Nitrate is commonly found in various dietary foods and in relatively high concentrations in green leafy vegetables as well as beetroots.  $\text{NO}_3^-$  can be reduced to nitrite ( $\text{NO}_2^-$ ) by oral bacteria, leading to an increased plasma nitrite concentration that serves as a circulating ‘reservoir’ for nitric oxide (NO) production (Lundberg and Govoni, 2004). NO is an important physiological signaling molecule that modulates skeletal muscle function through its role in the regulation of blood flow, muscle contractility, glucose and calcium homeostasis and mitochondrial biogenesis and respiration (Stamler and Meissner, 2001). In the past NO production was believed to only occur via an L-arginine mediated pathway. Only recently has it become apparent that NO may be produced by the reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  and subsequently  $\text{NO}_2^-$  to NO (Duncan et al., 1995) (see Figure 1.10).

The results from several studies have shown that the supplementation of dietary  $\text{NO}_3^-$  and the subsequent elevation of  $\text{NO}_2^-$  can result in reduced resting blood pressure and the submaximal  $\text{O}_2$  cost of exercise with subsequent improvements in performance (Bailey et al., 2010, Bailey et al., 2009, Lansley et al., 2011a, Lansley et al., 2011b, Vanhatalo et al., 2010). However, the proposed mechanisms behind these observations are still somewhat unclear. It has been suggested that the reduced  $\text{O}_2$  cost of exercise following dietary  $\text{NO}_3^-$  supplementation is related to a reduced ATP cost during muscle contraction possibly elicited through reduced cross-bridge cycling or sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase activity (Ferreira and Behnke, 2011). A second mechanism has also been proposed by Larsen et al., (2011) who have reported that  $\text{NaNO}_3^-$  reduced proton leakage which resulted in enhanced mitochondrial efficiency. Despite this observed reduction in oxygen cost and subsequent improved efficiency during submaximal exercise there is however a less clear translation to improved exercise performance (Jones et al., 2013)

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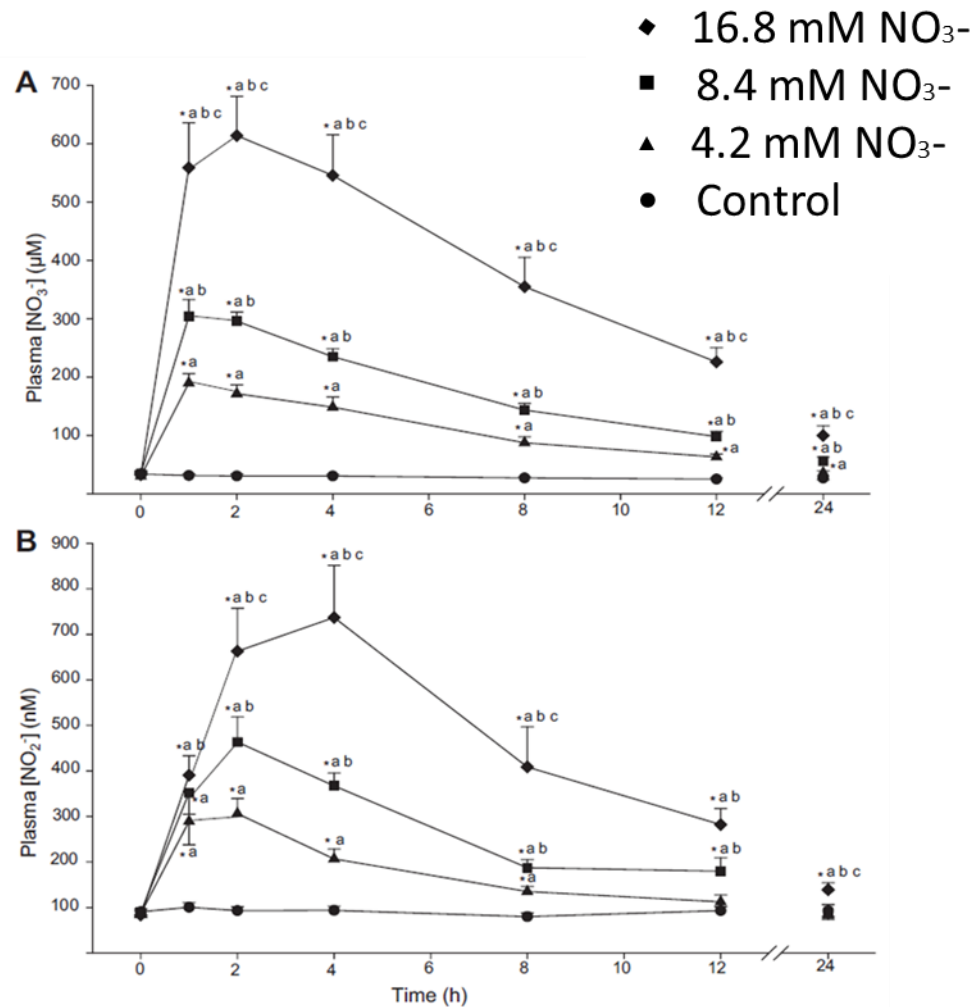


**Figure 1.10.** Reduction and proposed mechanisms of action for dietary nitrate. Dietary nitrate ( $\text{NO}_3^-$ ) is absorbed in the gastrointestinal system and concentrated in the saliva, where  $\text{NO}_3^-$  reducing bacteria convert  $\text{NO}_3^-$  to nitrite ( $\text{NO}_2^-$ ) which is absorbed and then circulates as a reservoir for nitric oxide ( $\text{NO}$ ) production. In the presence of hypoxia, ischemia and low pH as well as enzymes with nitrite reductase activity then reduce  $\text{NO}_2^-$  to  $\text{NO}$ . Through mechanisms thought to be related to mitochondrial efficiency and vasodilation, reductions in  $\text{O}_2$  cost during exercise as well as reduced blood pressure have been reported. Modified from Alef (2012).

#### 1.3.4.2 *Timing and dose*

Dietary supplementation with beetroot juice (BR), containing approximately 5–8 mM of inorganic  $\text{NO}_3^-$  has been shown to significantly increase plasma  $\text{NO}_2^-$  concentrations (Wylie et al., 2013) and evoke significant positive physiological responses as well as improve exercise performance in recreational (Lansley et al., 2011a, Murphy et al., 2012) and well-trained individuals (Bond et al., 2012, Cermak et al., 2012a). These responses have been observed after 3-6 day supplementation regimes as well as a single acute dose administered ~1-3 h prior to exercise. Recently, Wylie et al., (2013) investigated the dose-response relationship between the volume of  $\text{NO}_3^-$  ingested and its effects of performance. In that study 10 healthy recreationally active men ingested 70, 140, or 280 mL concentrated beetroot juice (containing 4.2, 8.4, and 16.8 mM  $\text{NO}_3^-$  respectively) to establish the effects on resting plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations over a 24 h period. Subsequently, on six separate occasions, 10 subjects completed moderate-intensity and severe-intensity cycle exercise tests, 2.5 h post-ingestion of 70, 140, and 280 mL of beetroot juice concentrate or an identical  $\text{NO}_3^-$ -depleted placebo. Following acute ingestion, plasma  $\text{NO}_2^-$  increased in a dose-dependent manner (shown in Figure 1.11), with the peak changes occurring at approximately 2–3 h. Compared with placebo, a 70 mL dose did not alter the physiological responses to exercise. However, 140 and 280 mL doses reduced the steady-state oxygen ( $\text{O}_2$ ) uptake during moderate intensity exercise by 1.7 and 3.0% whereas time-to-task failure was extended by 14% and 12% respectively, compared with placebo. The results indicate that whereas plasma  $\text{NO}_2^-$  concentration and the  $\text{O}_2$  cost of moderate-intensity exercise are altered dose dependently there is no additional improvement in exercise tolerance after ingesting beetroot juice concentrate containing 16.8 compared with 8.4 mM  $\text{NO}_3^-$ . However it has been noted that the apparent reduced effect of  $\text{NO}_3^-$  supplementation in well-trained individuals may

benefit from larger acute doses closer to that of 16.8 mM  $\text{NO}_3^-$  or possibly longer loading regimes in attempts to further increase circulating  $\text{NO}_2^-$  concentrations (Jones et al., 2013).



**Figure 1.11.** Plasma nitrate and nitrite concentration after varying doses of beetroot juice ingestion. Nitrate (A) and nitrite (B) concentrations after 4.2, 8.4 and 16.8 mM of  $\text{NO}_3^-$  from beetroot juice concentrate. A dose response is evident with peak concentrations occurring between 2-4 h post ingestion. \*Significant difference from pre-supplementation baseline ( $P < 0.05$ ); (a) significant difference from control ( $P < 0.05$ ); (b) significant difference from 4.2 mM  $\text{NO}_3^-$  ( $P < 0.05$ ); (c) significant difference from 8.4 mM  $\text{NO}_3^-$  ( $P < 0.05$ ); (Figure reproduced from Wylie et al. 2013)

#### *1.3.4.3 Nitrate supplementation and endurance performance*

The first study to implement the use of  $\text{NO}_3^-$  supplementation and report a reduced  $\text{O}_2$  cost during submaximal exercise was performed by Larsen et al., (2007). These findings raised great interest as it is well established that the  $\text{O}_2$  cost of exercise at a given submaximal power output is largely fixed, at least during cycling. These findings were corroborated by Bailey et al., (2009) who were the first to show that three days of beetroot juice supplementation increased plasma  $\text{NO}_2^-$  concentrations and reduced  $\text{O}_2$  cost during moderate intensity exercise as well as reduce the  $\dot{V} \text{O}_2$  'slow component' during severe-intensity exercise. The observed reduction in  $\text{O}_2$  cost after 3 days of  $\text{NO}_3^-$  supplementation by both Larsen et al., (2007) and Baily et al., (2009) was approximately 5% with similar effects also observed following acute  $\text{NaNO}_3$  intake ingested 60 min (Larsen et al., 2010) and beetroot juice supplementation 2.5 h (Vanhatalo et al., 2010) prior to exercise.

In spite of the observed enhanced 'metabolic efficiency' after acute and chronic supplementation of  $\text{NO}_3^-$ , the translation into enhanced performance in time to fatigue at constant load (TTF) as well as time trial performance appears to be somewhat less consistent. During TTF the improved exercise tolerance following  $\text{NO}_3^-$  supplementation has been reported to be in the range of 16-25% (Bailey et al., 2010, Bailey et al., 2009, Lansley et al., 2011a). However the magnitude of 'actual' sporting enhancement as represented by time trial performance is substantially less and is in the range of 1-4%. Table 1.3 displays the performance results of studies that have implemented  $\text{NO}_3^-$  supplementation in either a 3-6 day loading regime or as an acute dose prior to a time trial performance bout. It is hard to reach a consensus of findings due to the wide range of performance tasks, subject training history and supplementation regimes utilised within the investigations.

**Table 1.3.** Summary of the effects of acute or chronic nitrate supplementation on time trial performance.

Author	Year	Subjects	Nitrate Dose	Performance task	Improvement (p < 0.05)	Time (min)			Power (W)			Estimated VO2max
						CONT	NO <sub>3</sub> <sup>-</sup>	Δ	CONT	NO <sub>3</sub> <sup>-</sup>	Δ	
Lansley et al.	2011	Moderately trained cyclists	500ml BJ (~350mg NO3-) 75 min prior	4km Cycling TT	Yes	6.5	6.3	<b>+2.8%</b>	279	292	<b>4.6%</b>	98%
				16km Cycling TT	Yes	27.7	26.9	<b>+2.7%</b>	233	247	<b>6.0%</b>	95%
Cermak et al.	2012a	Well trained cyclists	140ml BJ concentrate (~500 mg NO3-) for 6 days	10km Cycling TT	Yes	16.1	15.8	<b>+1.2%</b>	288	294	<b>2.00%</b>	-
Cermak et al.	2012b	Well trained cyclists	140ml BJ concentrate (~550 mg NO3-) 150min prior	~1000 kJ Cycling TT	No	65.0	65.5	<b>-0.7%</b>	278	275	<b>-1.1%</b>	-
Wilkerson et al.	2012	Well trained cyclists	500 ml BJ (~380 mg NO <sub>3</sub> -) 150min prior	80km Cycling TT	No	137.9	136.7	<b>+0.8%</b>	238	235	<b>1.20%</b>	74%
Bescos et al.	2012	Well trained cyclists	10 mg/kg <sup>-1</sup> BM·day <sup>-1</sup> for 3 days (NaNO <sub>3</sub> -)	40 min cycle distance trial	No	-	-	-	257	258	<b>0.38%</b>	-
Bond et al.	2012	Well trained rowers	500 ml BJ (~340 mg NO <sub>3</sub> -) per day for 6 days	6 x 500 m Rowing TT	Yes	repetitions 1-6		<b>+0.4%</b>	-	-	-	100%
						repetitions 4-6		<b>+1.7%</b>				-
Murphy et al.	2012	Recreationally fit	200 g whole beetroot (~500 mg NO <sub>3</sub> -) 75min prior	5km Treadmill running TT	Yes	Running Speed (km/h)		<b>+3.3%</b>	-	-	-	-
Peacock et al.	2012	Well trained skiers	1 g KNO <sub>3</sub> <sup>-</sup> (614 mg NO <sub>3</sub> -) 150 min prior	5km running TT	No	16.6	16.75	<b>+0.9%</b>	-	-	-	-

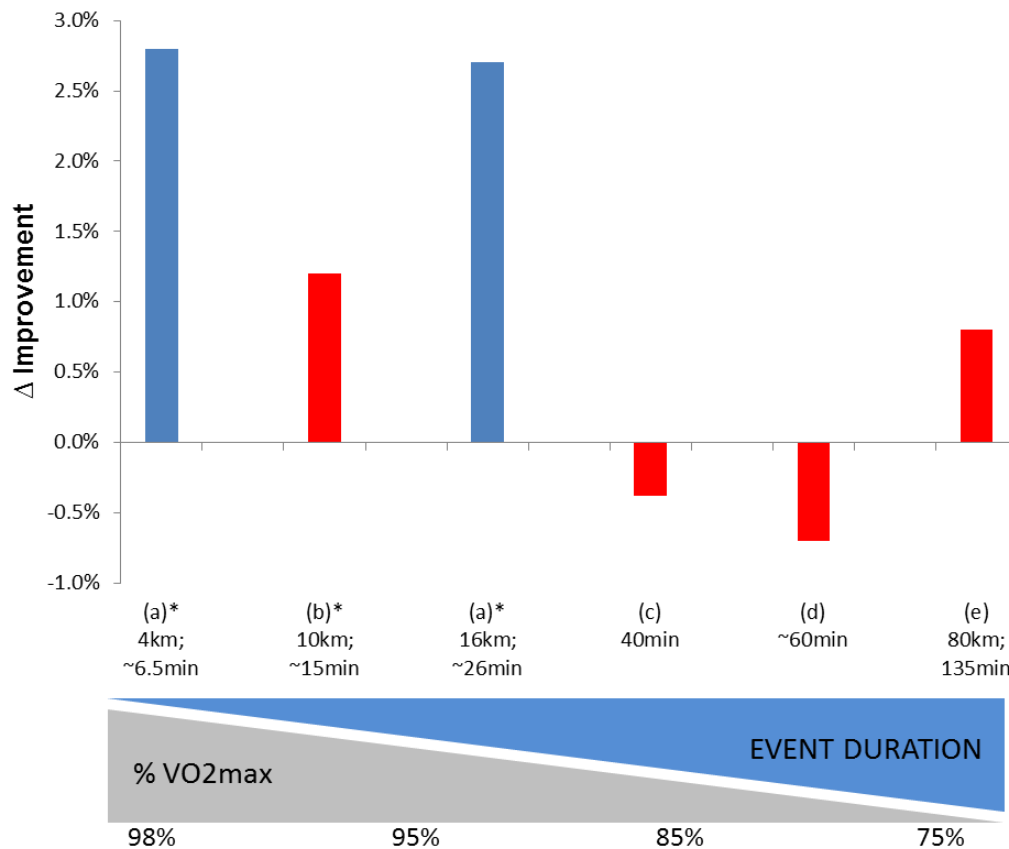
#### *1.3.4.4 Factors affecting the efficacy of nitrate supplementation*

The effects of  $\text{NO}_3^-$  supplementation appear to be more effective in time trial performance tasks requiring an individual to perform at a high relative percentage (85%) of maximal aerobic capacity. By nature of the proposed mechanisms of action for the ergogenic effect of  $\text{NO}_3^-$  supplementation (reduction in  $\text{O}_2$  cost) it makes sense that tasks performed at a greater percentage of  $\dot{V}\text{O}_{2\text{max}}$  may benefit to a greater extent when aerobic efficiency is improved, thereby resulting in a lesser reliance upon anaerobic metabolism

The trend toward greater improvement in performance after  $\text{NO}_3^-$  supplementation during cycling time trials of varying duration is displayed in Figure 1.12. The greatest performance improvements occurring in the 4km and 16km time trials in the study of Lansley et al., (2011a) was achieved after acute  $\text{NO}_3^-$  supplementation in moderately trained cyclists. The 10 km time trial from the study of Cermak et al., (2012a) who used well trained cyclist and a 6 day supplementation protocol did not achieve the same degree of performance improvement as that of Lansley et al., (2011a). To explain this observation it has been suggested that well trained athletes similar to those reported in the study of Cermak et al., (2012a) have higher resting  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations, which may reduce the scope for  $\text{NO}_3^-$  supplementation to improve efficiency and subsequent performance and may therefore require larger  $\text{NO}_3^-$  doses to elicit similar performance gains (Jones et al., 2013). Alternatively, more highly trained individuals who are likely to have greater mitochondrial and capillary density are less likely to experience the same degree of perturbation in acidosis and hypoxia during intense exercise. Therefore, the benefit of the proposed metabolic improvements afforded by the mechanisms of  $\text{NO}_3^-$  supplementation may not be attained to the same degree as that achieved in untrained individuals. Evidence for this inconsistency in effectiveness of  $\text{NO}_3^-$  supplementation within a well-trained cohort in comparison to recreationally fit individuals is further supported by comparing the studies of Murphy et al.,



(2012) and Peacock et al., (2012). Both studies investigated the effects of  $\text{NO}_3^-$  supplementation on a similar 5 km running TT. While Murphy et al., (2012) reported a ~3% performance improvement in recreationally active participants no significant performance enhancement was reported by Peacock et al (2012) in their well-trained participants.



**Figure 1.12.** Performance improvement after acute or chronic nitrate supplementation in well trained or moderately trained individuals. A possible effect of exercise intensity is evident with greater enhancements in performance reported during shorter more intense performance tasks. The training status of the individual has also been suggested to influence the efficacy of  $\text{NO}_3^-$  supplementation with untrained/recreational individuals responding to a greater extent compared to well-trained athletes; \* Studies reported significant performance improvements compared to placebo trial; blue bars represent studies with untrained/recreational cohort; red bars represent studies with a cohort of well-trained athletes. (a) Lansley et al., (2011), (b) Cermak et al., (2012a), (c) Bescos et al., (2012), (d) Cermak et al., (2012b), (e) Wilkerson et al., (2012)

#### *1.3.4.5 Summary*

Short-term dietary supplementation results in significant elevations in circulating  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations and appears to elicit several physiological benefits that have the ability to improve sporting performance. Current evidence suggests an ergogenic effect can be obtained for events requiring intensities of  $>85\% \dot{V} \text{O}_{2\text{max}}$  after 3-6 day supplementation or after acute ingestion of  $\text{NO}_3^-$  2-3 h prior to exercise, with evidence suggesting a greater improvement in untrained compared to trained individuals. Future investigations need to determine optimal dosing strategies as well as the identification of how the observed ergogenic effects if any, can translate to meaningful performance improvements in elite athletes.

### **1.3.5 Combining ergogenic aids**

To date, little research has investigated the potential effects of combining ergogenic aids with the intention of enhancing performance to a greater extent than when each is used in isolation. The premise behind this potential additive effect stems from the hypothesis that by combining two ergogenic aids that act through different mechanisms of action (i.e., central vs metabolic), the performance improvement would be greater than when each supplement is used in isolation. Caffeine supplementation offers a potential centrally acting supplement, while there are various metabolically acting ergogenic aids that could be co-ingested. The combination of caffeine with sodium bicarbonate (Carr et al., 2011, Kilding et al., 2012, Pruscino et al., 2008) or sodium citrate (Flueck et al., 2013) has been tested with the majority of findings suggesting no added benefit after co-ingestion. Pruscino et al., (2008) report a possible positive effect of the co-ingestion of caffeine and sodium bicarbonate during 2 x 200m swimming time trials, each separated by 30 min recovery. These workers concluded performance with caffeine alone, although displaying optimal performance in the first time trial subsequently led to reduced performance in the second trial, however, when combined with sodium bicarbonate, caffeine's negative impact on repeated maximal exercise performance was reversed (Pruscino et al., 2008).

Another potential combinational use of ergogenics is beta alanine and sodium bicarbonate. Sodium bicarbonate increases the extracellular buffering capacity (i.e.,  $H^+$  efflux from muscle cells to blood) by increasing the blood bicarbonate concentration, whereas the supplementation with beta alanine increases intracellular buffering capacity by augmenting intramuscular carnosine synthesis. To test this combination de Salles et al., (2013) investigated the effects of co-ingesting beta alanine and sodium bicarbonate on 200 m swimming time trial performance. These workers reported that compared with beta alanine

alone or a placebo the combination of beta alanine and sodium bicarbonate further increased 200 m swimming time trial performance (de Salles Painelli et al., 2013).

Another potential combination of ergogenics is the co-ingestion of dietary  $\text{NO}_3^-$  and caffeine. The improved skeletal muscle metabolic efficiency reported after  $\text{NO}_3^-$  ingestion combined with the centrally mediated improvement afforded after caffeine supplementation affords a likely additive effect. A recent study by Handzlik et al (2013) investigated this combination on performance during a time to exhaustion cycling trial at 80%  $\dot{V} \text{O}_{2\text{max}}$ . Fourteen well-trained cyclists ingested either caffeine (CAFF; 5  $\text{mg}\cdot\text{kg}^{-1}$  BM),  $\text{NO}_3^-$  (8 mM) or a placebo under four trial conditions consisting of CAFF+nitrate, CAFF+placebo,  $\text{NO}_3^-$  alone or placebo alone. Results revealed no significant differences between any trials however, optimal performance occurred after co-ingestion of CAFF+  $\text{NO}_3^-$  and that this was potentially due to a lower perceived effort during this trial. The co-ingestion of caffeine and  $\text{NO}_3^-$  delayed fatigue by 18 and 27% respectively compared to when  $\text{NO}_3^-$  and caffeine were ingested in isolation.

Collectively, these findings provide insight for future research to further investigate the potential combined use of ergogenic supplements. The wide range of sporting applications and potential ergogenic combinations affords vast possibility for future research. Other potential factors also include optimal dosing and timing for each supplement when co-ingested as well as the application in specific sports ranging from high intensity anaerobic events to prolonged endurance sports.

### ***1.4 Aims of this thesis***

It is clear that manipulation of CHO availability can augment training responses and subsequent training adaptation. However, performance during high intensity exercise is optimal when endogenous and exogenous CHO availability is high. Therefore, the implementation of ergogenic strategies that ‘rescue’ training intensity when exercise is purposefully commenced in a reduced glycogen state may afford added benefits to acute adaptive responses. There is also limited evidence regarding the efficacy of some newly emerging ergogenic aids under conditions of optimal or reduced CHO availability.

This thesis comprises four studies completed with the common theme of either enhancing performance or adaptation under conditions of optimal or sub-optimal CHO availability. The first two studies investigated ways to promote training adaptation under conditions of reduced muscle glycogen, specifically addressing the decline in training intensity observed when exercise is commenced with low muscle glycogen. The final two studies investigated the effectiveness of several ergogenic aids under conditions of either reduced or optimal CHO availability.

The aim of the first experimental study (Chapter 2) was to investigate caffeine ingestion as a possible strategy for ‘rescuing’ the aforementioned reduction in training intensity when high intensity exercise is commenced with reduced muscle glycogen availability. The hypothesis tested was that even under conditions of low glycogen availability, caffeine would increase maximal self-selected power output and thereby ‘rescue’ the reduction in training intensity observed when individuals commenced exercise with low glycogen availability.

While the first study aimed to ‘rescue’ the previously observed decline in training intensity in the face of muscle glycogen another possibility is to structure training so that quality high intensity training is completed when glycogen levels are high and a subsequent low intensity

training bout serves as the ‘train-low’ stimulus. Therefore, study two (Chapter 3) investigated the effects on acute cellular responses when feeding is withheld after high intensity exercise and a subsequent low intensity exercise bout is commenced with reduced muscle glycogen. The first exercise bout was performed in the evening with high CHO availability so that training intensity was not compromised. Then, athletes slept with reduced muscle glycogen availability and the following morning completed a second low intensity aerobic training session. It was hypothesised that by delaying feeding post exercise, selected signaling pathways and gene expression relating to training adaptation would remain elevated beyond that which normally occurs after feeding during acute recovery. Furthermore, by reversing the order of exercise (high intensity followed by low intensity compared to low intensity followed by high intensity as previously investigated) it was hypothesised that training intensity would not be compromised while the benefits of ‘train-low’ would still be achieved.

The previous two studies investigated possible ways to enhance training adaptation under conditions of reduced CHO availability. However, during competition an athlete’s priority shifts away from nutritional practices that promote adaptation, and toward strategies that optimise performance. The third study (Chapter 4) examined the effects of a CHO mouth rinse on cycling time trial performance commenced in either a fed or fasted state. Previous research has shown that a CHO mouth rinse is beneficial to high intensity exercise lasting <1 h eliciting an effect through a centrally mediated mechanism rather than metabolic. It was hypothesised that a CHO mouth rinse would be less effective when exercise was commenced in a fed state in comparison to a fasted state. These findings were aimed to be applied in competitive sporting situations where it is common for athletes to ingest a ‘pre-race’ meal prior to competition rather than commence exercise in an overnight fasted state as is common practice in laboratory controlled trials.

The final study (Chapter 5) investigated the independent and combined effects of caffeine and  $\text{NO}_3^-$  supplementation on performance during a cycling task commenced under nutritional conditions thought to be optimal to performance (eg, high CHO availability). This included the ingestion of a pre-event meal and regular oral CHO contact (eg, mouth rinse) during all trials. It was hypothesised that under optimal nutritional conditions: i) caffeine alone and ii)  $\text{NO}_3^-$  alone supplementation would improve time trial performance and iii) the concurrent use of caffeine and  $\text{NO}_3^-$  supplementation would result in an additive performance enhancement compared to when each supplement was used in isolation.



## ***Chapter Two***

### ***Caffeine ingestion and cycling power output in a low or normal muscle glycogen state***

This study is presented as the first in the series of related studies investigating the interactive effects of nutritional state and selected ergogenic aids upon cycling performance and training adaptation. Its purpose was to determine whether caffeine could ‘rescue’ the typically observed reduction in training intensity that occurs under conditions of low muscle glycogen.

The reference for the published version of this study is as follows:

**Lane, S. C., Areta, J. L., Bird, S. R., Coffey, V. G., Burke, L. M., Desbrow, B., Hawley, J. A.**  
(2013). *Med Sci Sports Exerc*, 45(8): 1577-84.

## *2.1 Introduction*

It has long been known that endurance training induces a many metabolic and morphological adaptations that improve the resistance of the trained musculature to fatigue and enhance endurance capacity and/or exercise performance (Holloszy, 1967). Accumulating evidence now suggests that many of these adaptations can be modified by nutrient availability (Hawley et al., 2011, Hawley and Burke, 2010, Hawley, 2011, Morton et al., 2009). Growing evidence suggests that training with reduced muscle glycogen using a ‘train twice every second day’ compared to a more traditional ‘train once daily’ approach can enhance the acute training response (Yeo et al., 2010) and markers representative of endurance training adaptation after short-term (3-10 wk) training interventions (Hansen et al., 2005, Hulston et al., 2010, Yeo et al., 2008b). Of note is that the superior training adaptation in these previous studies was attained despite a reduction in maximal self-selected power output (Hulston et al., 2010, Yeo et al., 2008b). The most obvious factor underlying the reduced intensity during a second training bout is the reduction in muscle glycogen availability. However, there is also the possibility that other metabolic and/or neural factors may be responsible for the power drop-off observed when two exercise bouts are performed in close proximity. Regardless of the precise mechanism(s), there remains the intriguing possibility that the magnitude of training adaptation previously reported in the face of a reduced training intensity (Hulston et al. 2010; Yeo et al. 2008b) might be further augmented, and/or other aspects of the training stimulus better preserved, if power output was not compromised.

Caffeine ingestion is a possible strategy that might ‘rescue’ the aforementioned reduction in power output that occurs when individuals commence HIT with low compared to normal glycogen availability. Recent evidence suggests that, at least in endurance-based events, the maximal benefits of caffeine are seen at small to moderate (2–3 mg·kg<sup>-1</sup> body mass [BM]) doses (for reviews see Burke 2008, Spriet 1995). Accordingly, the aims of this study were to

determine the effect of a low dose of caffeine ( $3 \text{ mg} \cdot \text{kg}^{-1} \text{ BM}$ ) on maximal self-selected power output during HIT commenced with either normal (NORM) or low (LOW) muscle glycogen availability. It was hypothesised that even under conditions of low glycogen availability caffeine would increase maximal self-selected power output and thereby partially rescue the reduction in training intensity observed when individuals commence HIT with low glycogen availability.

## **2.2 Methods**

### *Subject Characteristics*

Twelve competitive male endurance trained cyclists or triathletes cycling an average of  $263 \pm 51 \text{ km} \cdot \text{wk}^{-1}$  (range 210-400 km, all values mean  $\pm$ SD) in the 6 wk prior to commencement of the study and with a history of  $> 3$  yr endurance training volunteered to participate in this study. The subjects' age, body mass (BM), peak oxygen uptake ( $\dot{V}\text{O}_{2\text{peak}}$ ) and peak power output (PPO) were  $30.5 \pm 6$  yr,  $74.3 \pm 10.3$  kg,  $61.5 \pm 4.0 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  and  $369 \pm 31$  W. Prior to giving their written consent all subjects were informed of the possible risks of all procedures. The study was approved by the RMIT Human Research Ethics Committee.

### *Preliminary Testing*

One week prior to the first experimental trial all subjects undertook an incremental cycling test to exhaustion on an electronically braked cycle ergometer (Lode Excalibur *Sport*, Groningen, The Netherlands) as previously described (Hawley and Noakes, 1992). During this maximal test and parts of the subsequently described experimental trials, subjects breathed through a Hans Rudolph two-way non-rebreathing valve and mouth piece. This was attached to a calibrated online gas system (TrueOne 2400, Parvomedics, Utah, USA)

interfaced to a computer that calculated the instantaneous rates of  $\text{O}_2$  consumption ( $\dot{V}\text{O}_2$ ),  $\text{CO}_2$  production ( $\dot{V}\text{CO}_2$ ), minute ventilation ( $\text{VE}_{\text{STPD}}$ ), and the respiratory exchange ratio (RER). Before each maximal test and all experimental trials the analysers were calibrated with commercially available gasses of known  $\text{O}_2$  and  $\text{CO}_2$  content.  $\dot{V}\text{O}_{2\text{peak}}$  was defined as the highest  $\text{O}_2$  uptake a subject attained during any 30 s of the test while PPO was calculated from the last completed work rate plus the fraction of time spent in the final non-completed work rate as previously described (Hawley and Noakes, 1992). This value was used to determine the power output corresponding to 70% of each subjects'  $\dot{V}\text{O}_{2\text{peak}}$  (~63% PPO) to be used in the experimental trials. The maximal test and all experimental trials were conducted under standard laboratory conditions (18-22°C, 40-50% relative humidity), and subjects were fan cooled during all exercise sessions.

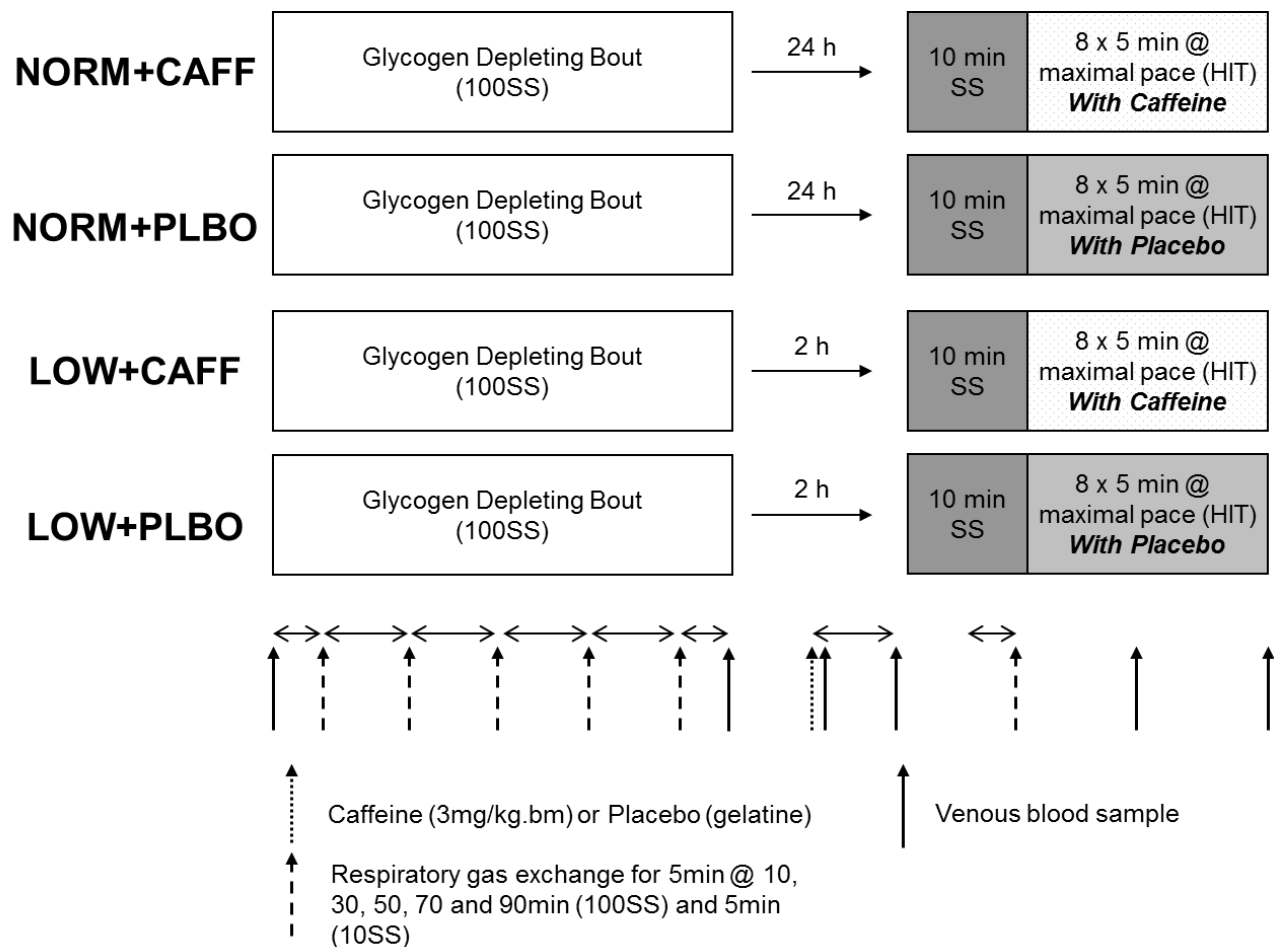
#### *Familiarisation to High Intensity Interval Sessions*

After completing the maximal test, subjects rested for ~20 min before undertaking a familiarisation trial. Using their own bicycles mounted on a stationary trainer (Kurt Kinetic, Minnesota, USA) each subject performed between four and six 5 min work-bouts with 60 s recovery, at their maximal self-selected power output. Power output was monitored using a PowerTap power meter (CycleOps, Saris Cycling Group, Wisconsin, USA). Prior to each familiarisation session, and all experimental trials the power meter was set to zero (reset) as per the manufacturer's instructions. Subjects were subsequently instructed to select the highest power output they believed they could sustain for 8 x 5 min work-bouts: if any subject failed to repeatedly produce power outputs of > 70% of their PPO for the final three work-bouts they were required to return to the laboratory and undertake another

familiarisation ride until the principal investigator deemed the subject capable of reproducing consistent power outputs during the subsequent experimental trials.

### *Overview of the Experimental design*

A schematic overview of the experimental protocol is shown in Figure 2.1. In brief, subjects completed four experimental trials each separated by ~7 days. Trials were completed using a Latin Square design double blinded for caffeine and placebo. Two experiments were commenced under conditions of NORM and two under conditions of LOW glycogen availability. Pre-trial glycogen levels were manipulated by diet-exercise interventions as previously validated by this laboratory using direct measures of muscle glycogen (Yeo et al., 2008b). For all experimental trials, subjects reported to the laboratory between 0700-0730 h after a 9-10 h overnight fast and a standardised diet (detailed subsequently). Subjects then rested quietly for 15 min before a teflon catheter (Terumo, 20-22G, Tokyo, Japan) was inserted into a vein in the antecubital fossa to allow for repeated blood sampling. Either caffeine ( $3 \text{ mg} \cdot \text{kg}^{-1} \text{ BM}$ ) or a placebo was administered 60 min prior to all experimental trials which consisted of a 10 min warm up at 63% of PPO (10SS) followed by HIT



**Figure 2.1.** Experimental trials: NORM+CAFF (Normal Glycogen levels with Caffeine); NORM+PLBO (Normal Glycogen levels with Placebo); LOW+CAFF (Low Glycogen levels with Caffeine); LOW+PLBO (Low Glycogen levels with Placebo); 100SS (100 min Steady State at 63% Peak Power Output); 10SS (10 min Steady State at 63% Peak Power Output immediately prior to HIT); HIT (High Intensity Interval Training).

### *Diet/Exercise control*

Subjects refrained from all strenuous physical activity and both caffeine and alcohol intake during the 48 h before an experimental trial. A 48 h caffeine withdrawal period has previously been shown to restore the hormonal response during exercise in habitual user's commensurate to that of non-users (Van Soeren and Graham, 1998). Each subjects' diet was 'clamped' 24 h before each experiment at an energy intake of  $0.21 \text{ MJ} \cdot \text{kg}^{-1} \text{ BM} \cdot \text{day}^{-1}$  ( $8 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  of energy from CHO;  $2.0 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  from protein and  $1.0 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  from fat). All meals and snacks were supplied to subjects with diets individualised for food preferences and BM. Subjects received their food in pre-prepared packages and were required to keep a food checklist to note their compliance to the dietary instructions and their intake of any additional food or drinks. During the 24 h recovery period prior to a NORM trial subjects refrained from any physical activity and were provided with an identical high CHO diet (as previously described above) to ensure adequate glycogen repletion prior to commencement of the experimental trials. To ensure both LOW trials were commenced with reduced muscle glycogen availability, subjects only consumed water during the 2 h period between the glycogen depleting session and the experimental trial (Yeo et al., 2008b). Subjects were allowed water *ad libitum* during all rides.

### *Glycogen depletion session*

Either 24 (NORM) or 2 h (LOW) prior to the experimental trials subjects cycled on a Lode ergometer at an intensity of 63% of PPO ( $\sim 70\% \dot{V} \text{O}_{2\text{peak}}$ ) for 100 min (100SS). This intensity and duration has previously been shown to deplete muscle glycogen by 50% in well-trained cyclists (Yeo et al., 2008b, Coyle et al., 1986). During the 100SS, respiratory data, heart rate (HR) and rating of perceived exertion (RPE) were collected at 10, 30, 50, 70 and 90 min. Each

sampling time point was recorded as a 5 min average. Each subject's HR was recorded using a wireless heart rate monitor (Polar, Kempele, Finland) and perceived exertion was recorded using the Borg scale of 6-20 (Scherr et al., 2013).

### *Caffeine and placebo administration*

Subjects ingested an opaque capsule 60 min prior to the commencement of each experimental trial (Figure 2.1). The capsule contained either 3 mg·kg<sup>-1</sup> BM of anhydrous caffeine (CAFF; #30-1158, PCCA, Matraville, NSW, AUS) or a matched placebo (PLBO; Gelatine) depending on that day's experiment. Subjects consumed each capsule with 200 mL of water.

### *Experimental session*

Each experimental trial consisted of a 10 min warm-up (10SS) undertaken at the same intensity (63% PPO) as each subject's 100SS ride. Respiratory and HR data were collected as an average for the final 5 min of exercise, while RPE was recorded as a single value in the final minute of the 10SS ride. Upon completion of the warm-up, subjects immediately mounted their own bicycles, which were attached to a stationary trainer. Subjects cycled for ~2 min at a workload of < 150W prior to the commencement of HIT (8 x 5 min repetitions at maximal self-selected intensity with 1 min recovery). During the experimental trials subjects received no external feedback or motivation other than elapsed time during each interval and the number of remaining intervals. Power output (W) was monitored using a PowerTap power meter, calibrated as previously described. Mean HR, power output, cadence and RPE were recorded at the completion of each interval.



### *Blood samples*

A total of 12 mL of whole blood was obtained at each sampling time point. Four mL of whole blood was collected in tubes containing EDTA of which 25  $\mu$ L were immediately analysed for glucose and lactate concentrations (YSI, Yellow Springs, Ohio, USA). Four mL of whole blood was collected in a tube containing lithium heparin for determination of plasma caffeine concentration while 3 mL were collected in a tube containing EGTA for analyses of plasma free fatty acid (FFA) concentration. Samples were stored on ice and then centrifuged at 4°C at 4000 rev·min<sup>-1</sup> (3040 x g) for 10 min. The resultant plasma for both caffeine and FFA analyses was transferred to empty 1.5 mL storage tubes. Plasma FFA concentrations were later determined using an enzymatic colorimetric method (NEFAC code 279-75409, Wako, Tokyo Japan). Plasma was stored at -80 °C until analyses were undertaken.

### *Plasma caffeine concentration*

The quantitative analysis of plasma caffeine was performed using an automated ‘reverse phase’ high-performance liquid chromatography system. Conditions were adapted with subtle modifications from Koch (1999). The precise method has previously been described by Desbrow et al. (2009)

### *Statistical Analysis*

Statistical analyses were performed using software package SPSS (Version 18). Main effects of caffeine and glycogen were analysed using two-way repeated measures ANOVA’s. For all blood and physiological measures one-way ANOVA’s for repeated measures were used to

compare between time points and trials using a Bonferroni adjustment where appropriate. Mean power output from the four trials were analysed using the magnitude based inference approach recommended for studies in sports medicine and exercise (Hopkins et al., 2009). A spreadsheet (Microsoft Excel), designed to examine post-only crossover trials, was used to determine the clinical significance of each treatment (available at [newstats.org/PostOnlyCrossover.xls](http://newstats.org/PostOnlyCrossover.xls)), as based on guidelines outlined by Hopkins (2007). Qualitative inferences are reported as the percentage chance of a positive effect compared to the corresponding trial where a least worthwhile effect on power output of 1% was used as previously established (Paton and Hopkins, 2006). Significance was set at  $P < 0.05$ . All data are presented as mean  $\pm$ SEM unless otherwise stated.

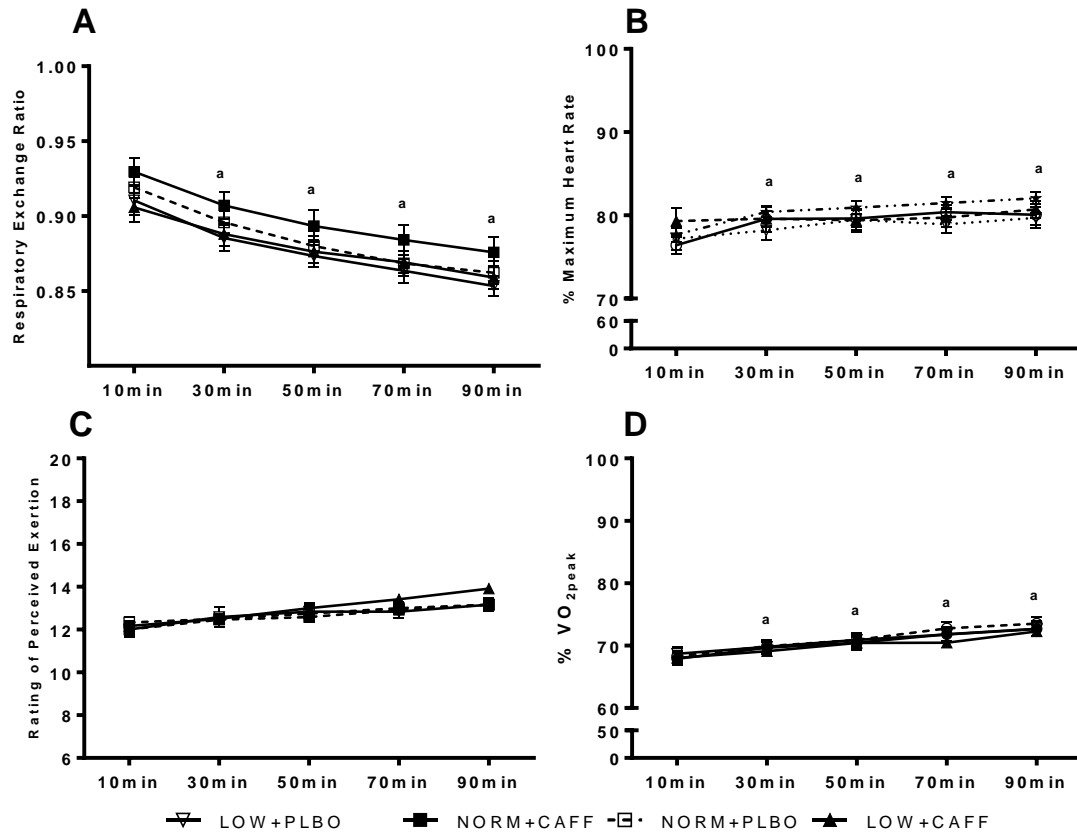
## 2.3 Results

### *Habitual caffeine intake*

Using a recall questionnaire the self-reported habitual mean caffeine intake of subjects prior to the preliminary testing was  $154 \pm 89 \text{ mg} \cdot \text{day}^{-1}$  (range 10 to 294  $\text{mg} \cdot \text{day}^{-1}$ , mean  $\pm$ SD ).

### *Glycogen depletion sessions (100SS)*

There were no significant differences between trials for HR,  $\dot{V} \text{O}_2$ , RER or RPE at any time point during the 100SS (Figure 2.2). Compared to the 10 min time point there was a time dependent reduction in RER and an increase in HR and  $\dot{V} \text{O}_2$  for all trials ( $P < 0.05$ ).

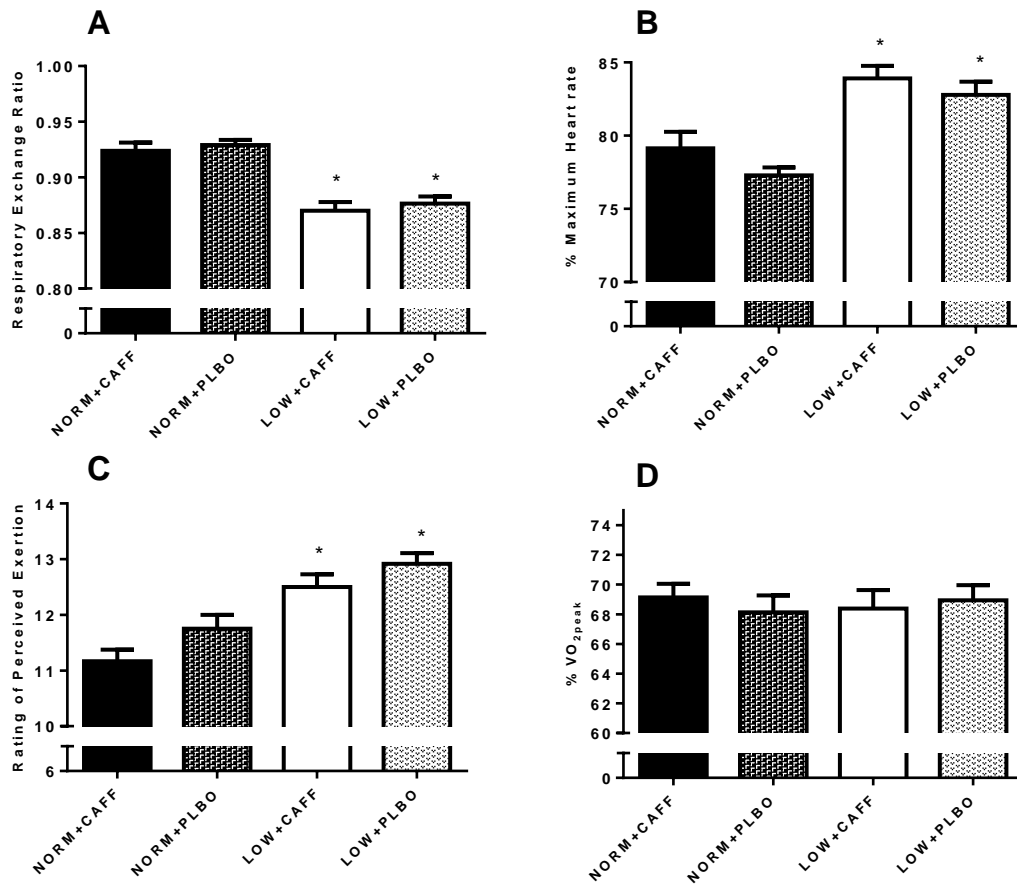


**Figure 2.2.** Physiological characteristics during the 100 min steady state cycling bout: A) RER, B) % Maximum Heart Rate, C) Rating of Perceived Exertion, D) %  $\dot{V}O_{2peak}$ . RER; Respiratory Exchange Ratio, RPE; Rating of Perceived Exertion, %  $\dot{V}O_{2peak}$ ; Percent of Maximal oxygen uptake. Values are expressed as means  $\pm$ SEM. (a) significantly different to 10 min for all trials ( $P < 0.05$ ).

## *Experimental Sessions*

### *10min Steady State (10SS)*

Figure 2.3 displays the effects of glycogen availability and caffeine on physiological variables during the 10SS bout. There was a main effect of glycogen availability (NORM vs. LOW) on HR, RPE and RER during the 10SS bout ( $P < 0.001$ ). There was a trend for caffeine to reduce RPE ( $P = 0.06$ ) and increase HR ( $P = 0.07$ ) but no main effect for caffeine was detected on any other variable.

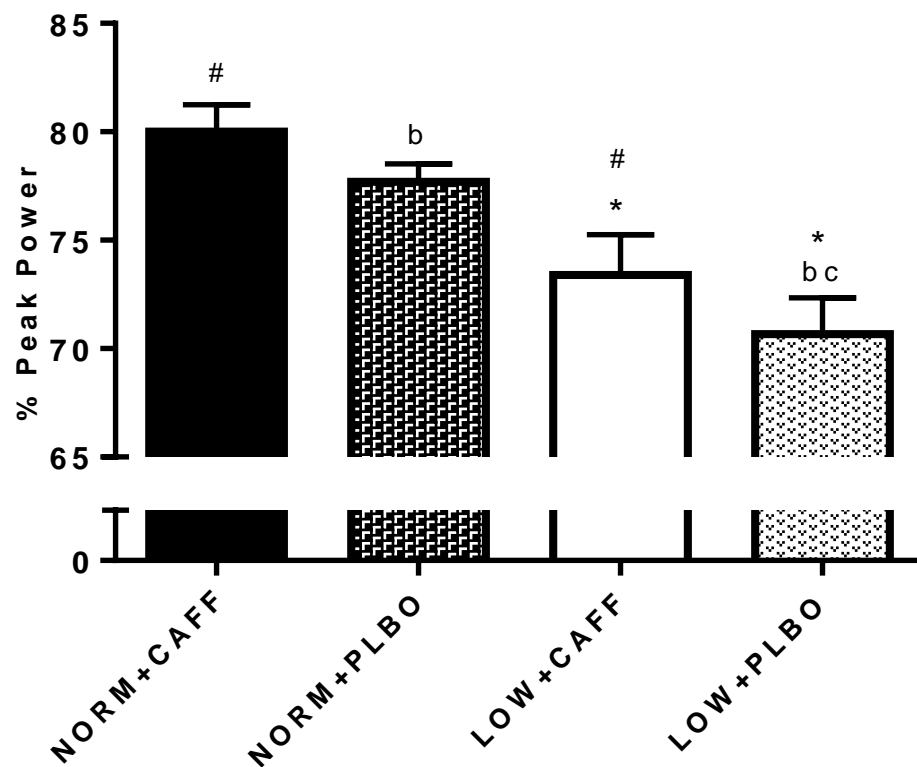


**Figure 2.3.** Physiological characteristics during the 10min steady state cycling bout: A) RER, B) % Maximum Heart Rate, C) Rating of Perceived Exertion, D) %  $\dot{V}O_{2peak}$ . Values are expressed as means  $\pm$ SEM. (\*) significant main effect of muscle glycogen levels ( $P < 0.001$ ).

### *High Intensity Intervals (HIT)*

#### *Power Output*

There was a main effect ( $P < 0.01$ ) for both glycogen availability and caffeine on mean power output (Figure 2.4), but no interaction. Commencing HIT with low muscle glycogen availability reduced mean power output by 8.1% (LOW+CAFF) and 8.6% (LOW+PLBO) compared to the corresponding NORM trials. There was a difference in mean power output between NORM+CAFF and NORM+PLBO (2.8%,  $P = 0.01$ ) while a similar effect was evident for the LOW+CAFF and LOW+PLBO trials (3.5%,  $P = 0.05$ ). Using the qualitative inference approach there was a ‘very likely’ positive effect (97%) of caffeine on power output during the NORM trials and a ‘likely’ positive effect (92%) of caffeine during the LOW trials. Caffeine failed to fully restore self-selected intensity (LOW+CAFF vs. NORM+PLBO) as there was a significant deficit in power output between these trials of 5.1% ( $P = 0.036$ ).



**Figure 2.4.** Mean percentage of peak power output during High Intensity Intervals (HIT): NORM+CAFF, (Normal glycogen levels with caffeine); NORM+PLBO, (Normal glycogen levels with placebo); LOW+CAFF, (Low glycogen levels with caffeine); LOW+PLBO, (Low glycogen levels with placebo). Values are means  $\pm$ SEM. (b) significantly different to NORM+CAFF ( $P < 0.05$ ), (c) significantly different to NORM+CAFF, NORM+PLBO ( $P < 0.05$ ), (\*) significant main effect of muscle glycogen levels, (#) significant main effect of caffeine.



### *Heart Rate*

A main effect ( $P < 0.001$ ) revealed that HR was higher in the CAFF when compared to the PLBO trials. When each CAFF trial was compared to its corresponding PLBO trial this trend remained, but did not attain significance (NORM+CAFF vs. NORM+PLBO;  $90 \pm 0.8\%$  vs.  $88 \pm 0.1\%$ ,  $P = 0.1$ , LOW+CAFF vs. LOW+PLBO;  $90 \pm 0.1\%$  vs.  $88 \pm 0.8\%$ ,  $P = 0.1$ ).

### *Cadence*

There was a main effect for both muscle glycogen levels and caffeine ingestion on self-selected cadence ( $P < 0.01$ ). Cadence was greater during NORM+CAFF ( $99 \pm 2 \text{ rev}\cdot\text{min}^{-1}$ ) compared to all other trials (NORM+PLBO,  $94 \pm 2 \text{ rev}\cdot\text{min}^{-1}$   $P < 0.01$ ; LOW+CAFF,  $93 \pm 2 \text{ rev}\cdot\text{min}^{-1}$ ,  $P < 0.05$ ; LOW+PLBO,  $92 \pm 2 \text{ rev}\cdot\text{min}^{-1}$ ,  $P < 0.01$ ).

### *Blood glucose concentration*

There was a main effect of muscle glycogen levels (NORM vs. LOW) on blood glucose concentration at all time points after the 100SS ride ( $P < 0.05$ ; Figure 2.5A). There was a time-effect during the HIT session during NORM+CAFF in which blood glucose was higher MIDHIT ( $5.5 \pm 0.3 \text{ mM}\cdot\text{L}^{-1}$ ,  $P < 0.05$ ) and POSTHIT ( $6.7 \pm 0.5 \text{ mM}\cdot\text{L}^{-1}$ ,  $P < 0.01$ ) when compared to PREHIT ( $4.4 \pm 0.1 \text{ mM}\cdot\text{L}^{-1}$ ).

### *Blood lactate concentration*

Figure 2.5B displays the blood lactate concentrations during all trials. There was a main effect of muscle glycogen on lactate concentration at MIDHIT ( $P < 0.05$ ) and POSTHIT ( $P < 0.01$ ). A main effect of caffeine ingestion on blood lactate concentrations was observed at PRE, MID and POSTHIT ( $P < 0.01$ ). Blood lactate concentrations were greater MIDHIT and POSTHIT for all trials when compared to all other times points ( $P < 0.01$ ).

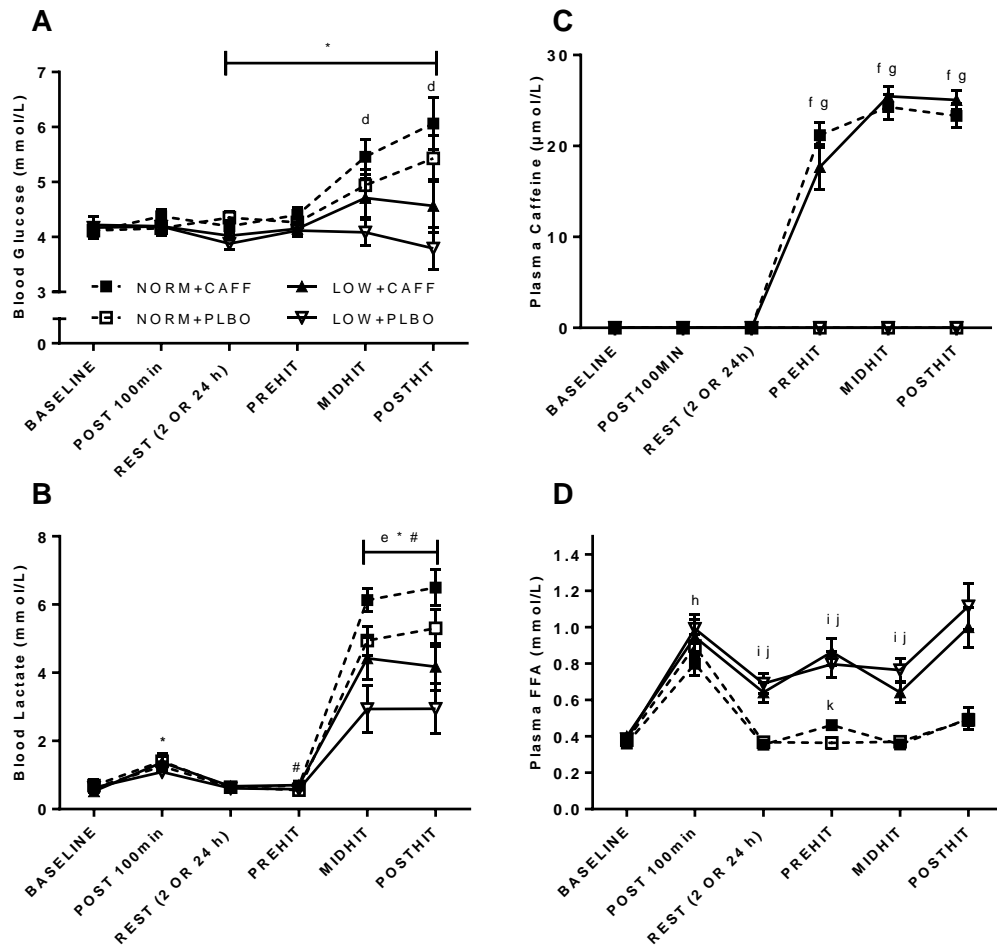
### *Plasma caffeine concentrations*

Figure 2.5C shows the plasma caffeine concentrations for all trials. Resting plasma caffeine concentrations revealed that all subjects complied to the dietary guidelines and abstained from consuming any caffeine containing products in the 48 h prior to all experimental trials. Plasma caffeine concentrations peaked at MIDHIT (HI+CAFF  $24.3 \pm 1.4$ , LOW+CAFF  $25.4 \pm 1.0 \mu\text{mol}\cdot\text{L}^{-1}$ ) and remained elevated at the completion of HIT. Plasma caffeine concentration was greater in NORM+CAFF and LOW+CAFF at PREHIT, MIDHIT and POSTHIT compared to their respective PLBO trials ( $P < 0.01$ ).

### *Plasma free fatty acid concentration*

Figure 2.5D shows the plasma FFA concentrations throughout all trials. Immediately post the 100SS ride plasma FFA concentrations were significantly increased ( $P < 0.01$ ) in all trials. Prior to the NORM experimental trials FFA concentrations returned to baseline after the 24 h recovery period (after consuming a high CHO diet). However, during the NORM+CAFF trial FFA concentrations were elevated at 60 min post CAFF ingestion at PREHIT compared to

NORM+PLBO ( $P < 0.01$ ). Prior to the LOW experimental trials plasma FFA concentrations remained elevated compared to BASELINE ( $P < 0.01$ ) and were elevated at all time points when compared to NORM trials ( $P < 0.05$ ).



**Figure 2.5.** Metabolic responses during trails. A) Blood glucose concentration, B) Blood lactate concentration C) Plasma caffeine concentration D) Plasma Free Fatty Acid concentration: Values are means  $\pm$ SEM. (\*) significant main effect of muscle glycogen levels ( $P < 0.01$ ), (#) Significant main effect of caffeine (e) significantly different to PREHIT in NORM+CAFF only ( $P < 0.05$ ), (f) significantly different to all time points prior to MIDHIT for all trials ( $P < 0.01$ ), (g) significantly different to REST (2h OR 24h) in CAFF trials ( $P < 0.01$ ), (h) significantly different to corresponding time points in PLBO trials ( $P < 0.01$ ), (i) significantly different to BASELINE for all trials ( $P < 0.01$ ), (j) significantly different to BASELINE for LOW trials ( $P < 0.01$ ), (k) significantly different to corresponding time point in NORM trials ( $P < 0.01$ ), (l) significantly different to NORM+PLBO ( $P < 0.05$ ).

## 2.4 Discussion

The aim of this study was to determine the effect of ingesting a low dose of caffeine ( $3\text{mg}\cdot\text{kg}^{-1}$  BM) on maximal self-selected power output during HIT commenced with either normal or low muscle glycogen availability. The original hypothesis was that even under conditions of low glycogen availability, caffeine ingestion would enable the well-trained subjects to partially restore power output closer to the intensities attained when the same workouts were commenced with normal glycogen levels. The proposed performance benefits of caffeine ingestion ascribe a centrally mediated effect as previously reported. A novel finding was that independent of glycogen availability, caffeine enhanced work capacity during intense interval training by approximately 3%. However, the ergogenic effect of caffeine was insufficient to completely ‘rescue’ the decrease in power output attributable to low glycogen availability. The finding of a ~3% improvement in power output is consistent with other studies that have reported an enhanced work capacity when caffeine ( $6\text{--}9\text{ mg}\cdot\text{kg}^{-1}$  BM) was ingested in the hour prior to either short duration (~5 min) maximal exercise (Anderson et al., 2000, Bruce et al., 2000) and cycling time trial performance lasting ~1 h (McNaughton et al., 2008). The results also provide further support to previous findings (Jenkins et al., 2008) that low doses of caffeine ( $2\text{--}3\text{ mg}\cdot\text{kg}^{-1}$  BM) provide an ergogenic benefit (~3-4%) during high intensity cycling. Notably, the self-selected pedal velocity (cadence) by subjects during all trials corresponded to power output during the performance trials where, NORM+CAFF was significantly higher (~99rpm) than all other trials (92-94rpm). Given that subjects were instructed to self-select their own cadence during HIT it appears that the reduced perceived exertion reported after caffeine ingestion allowed the subjects to self-select a higher cadence as opposed to generating greater force at a lower cadence.

Commencing HIT with reduced glycogen availability reduced mean power output by ~8%, a figure that is in close agreement with the power drop-off previously reported by both Yeo et

al., (2010) and Hulston et al., (2010) during similar HIT sessions commenced 1-2 hours after glycogen depleting cycling. It is interesting to hypothesise that the observed partial rescue in power output after caffeine ingestion in the current study could further enhance the already augmented training response that occurs when selected sessions are commenced with reduced glycogen availability: such a hypothesis would need to be tested with long-term training studies. Additionally the findings of the current study reinforce the viewpoint that low doses of caffeine are capable of significantly improving performance during competition that requires athletes to compete in multiple endurance type events in close succession.

An assumption of the present study was that the subjects commenced the LOW trials with substantially lower (~50%) muscle glycogen availability than the NORM trials. Muscle biopsies were not collected to measure pre-HIT muscle glycogen concentrations in this study as this may have adversely affected HIT cycling performance. Previous data would suggest that the LOW trial was commenced with significantly reduced muscle glycogen concentrations. Yeo et al., (2008b) using a similar cohort of athletes completed the exact same exercise-diet regimen and demonstrated that it produced resting muscle glycogen concentrations between the LOW and NORM conditions that differed by ~50%. Further support of a likely reduction in muscle glycogen availability in the LOW conditions comes from the elevated FFA concentrations immediately prior to commencing HIT (0.8 vs. 0.4 mM·L<sup>-1</sup>), and the lower RER (0.87 vs. 0.94) during the warm-up (10SS) prior to commencing the LOW vs. NORM trials. Such evidence supports that all LOW experimental trials were commenced with reduced muscle glycogen availability and, conversely, that the 24 h period of high-CHO intake (8 g·kg<sup>-1</sup> BM of CHO) and rest was adequate to restore muscle glycogen to normal levels during the NORM trials. It has previously been shown that when training intensity is 'clamped' at ~82% of PPO (~86% of  $\dot{V}O_{2\text{ peak}}$ ) for 8 x 5 min work-bouts, muscle glycogen content declines by 50% of resting values (Stepto et al., 2001). Taken together with

the 50% reduction in glycogen content previously reported by Yeo et al., (2008b) following 100 min of cycling at 63% PPO, it can be hypothesised that glycogen concentrations at the end of HIT in the LOW trials would be almost entirely depleted.

There were significant differences between the NORM and LOW trials in respect to blood glucose concentrations during and post the HIT sessions. Blood glucose concentrations tended to rise during the HIT session of the NORM trials suggesting adequate liver glycogen availability to maintain blood glucose and also to sustain the high rates of glucose uptake by working skeletal muscle. Conversely, blood glucose concentrations during the LOW trials when CHO availability was compromised (presumably within both skeletal muscle and the liver) approached levels of hypoglycaemia ( $<4 \text{ mM}\cdot\text{L}^{-1}$ ). The reduction in self-selected intensity during the LOW trials may be multifactorial. Firstly, central mechanisms monitoring reduced whole body CHO availability (muscle, liver and blood) may stimulate protective mechanisms, which result in a reduction in central output. Secondly, inadequate intramuscular and blood glucose availability would result in the inability to sustain high rates of glycolysis within working skeletal muscle.

The magnitude of reduction in self-selected training intensity reported in the current study (~8%) and by that of Yeo et al., (2010) and Hultson et al., (2010) under conditions of reduced glycogen availability is somewhat perplexing in light of previous findings. Stepto et al., (2002) reported that six of seven well-trained cyclists/triathletes were able to complete an identical HIT bout (8 x 5 min) with intensity clamped at ~82% PPO after 4 days of a high-fat low CHO diet, which has been shown to reduced pre-exercise muscle glycogen concentrations to  $\sim 255 \text{ mM}\cdot\text{kg}^{-1}$  dry wt (Burke et al., 2000). In the present study, only during the NORM+CAFF trial was power output close (~80% of PPO) to the value employed in the Stepto et al., (2002) investigation. As the subjects in the study of Stepto et al., (2002) were of

similar training status to those of the current study this suggests that the 'maximal' self-selected power output chosen by the current subjects may be lower than a work rate that is physiologically sustainable. Taken collectively, the results of the present study along with previous findings (Stephens et al., 2001, Stephens et al., 2002, Yeo et al., 2010) suggest that the reduction in self-selected training intensity during HIT could be due to a combination of factors and not just a lack of CHO availability. One possibility of course, is the residual fatigue associated with two training sessions undertaken in close proximity.

Caffeine ingestion was able to augment plasma FFA concentrations even in the face of low glycogen availability, a condition in which they were already substantially elevated (Figure 2.5D). Chronic elevations in FFA availability has been shown to increase mitochondrial markers in rats (Fillmore et al., 2010) and improve fat oxidation while sparing muscle glycogen during exercise in humans (Yeo et al., 2008a). While commencing exercise with reduced muscle glycogen availability appears to have a stimulatory effect on several cell signaling pathways involved in energy sensing (McBride et al., 2009) it is not yet known if it is in fact reduced muscle glycogen availability or the concomitant elevation in circulating FFAs that are responsible for the acute signaling (Yeo et al., 2010) and chronic upregulation of oxidative markers after 'train-low' interventions (Yeo et al., 2008b). Further investigations are required to fully understand the precise mechanisms responsible for the enhanced adaptation and to determine whether it is a direct result of training with low glycogen availability, higher FFA levels, a combination of both or simply performing two training sessions twice in close proximity.

In conclusion this study has demonstrated that caffeine enhances power output independently of muscle glycogen availability. However, caffeine at low doses ( $3 \text{ mg}^{-1} \cdot \text{kg}^{-1} \text{ BM}$ ) cannot fully restore maximal self-selected power output during high-intensity interval training to



those levels attained when well-trained athletes commence the same sessions with normal muscle glycogen concentration. Whether the small but significant caffeine-induced increase in power output observed in the face of low glycogen availability can augment training adaptation when performed chronically (i.e. over a 4-6 wk training cycle) remains to be determined.

### *2.5 Acknowledgments*

The hard work and commitment of the athletes who gave their time to participate in this research project was greatly appreciated. This study would also not have been possible without the assistance of everyone from the RMIT Exercise and Nutrition Research Group who assisted in running the trials. Ben Desbrow is also acknowledged for his contribution for the analysis of plasma caffeine concentrations.



# ***Chapter Three***

## ***The effects of delayed post-exercise feeding on acute metabolic responses and a subsequent exercise bout***

Previous research has shown that molecular responses to training are superior when subjects commence selected training sessions with low glycogen levels. The superior response/adaptations are elicited despite the fact that the reported training intensity is ~8% lower under conditions of low compared to high glycogen availability. The previous chapter determined if caffeine could restore the reduction in power output observed when training under conditions of low glycogen concentrations and whether this afforded a means to further magnify the training response. Another means to preserve training intensity is to manipulate the order of exercise and feeding. Whereas past research has used prolonged endurance exercise to deplete glycogen followed by a subsequent HIT bout, the present study investigated the effects on metabolic and early gene responses of first completing HIT, which served to reduce muscle glycogen, while preserving training intensity, followed by a subsequent low intensity aerobic training session. By undertaking HIT first this circumvented the reduction in training intensity whereas, the low intensity of the subsequent aerobic session would be less affected when commenced with reduced glycogen availability. The study design also determined the effects of withholding feeding post an evening HIT bout and subsequently sleeping with reduced glycogen content upon acute metabolic and cellular responses.

The manuscript for this study is still in preparation:

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### ***3.1 Introduction***

Changes in macronutrient intake rapidly alter the concentration of circulating substrates and hormones, causing marked perturbations in the storage profile of skeletal muscle and other insulin-sensitive tissues. Subsequently, muscle energy status exerts profound effects on patterns of fuel oxidation during exercise, as well as many of the acute regulatory processes underlying gene expression and cell signaling. As such, nutrient-exercise interactions have the potential to activate or inhibit many biochemical pathways with putative roles in training adaptation (Hawley et al., 2011).

Commencing endurance exercise with low muscle glycogen stores (so-called “train-low”) results in a greater transcriptional activation of enzymes involved in carbohydrate metabolism, including the adenosine 5'-monophosphate-activated protein kinase (AMPK), GLUT4 and the pyruvate dehydrogenase (PDH) complex, compared with when glycogen content is normal (Cochran et al., 2010, Pilegaard et al., 2002, Wojtaszewski et al., 2003). Restricting carbohydrate availability during early (1-5 h) post-exercise recovery has also been shown to acutely up-regulate various markers of substrate metabolism and endurance training adaptation in skeletal muscle (Pilegaard et al., 2005, Cochran et al., 2010). Because the time course of transcriptional activation for many exercise-induced genes occurs during the first few hours of recovery (Mahoney et al., 2005), returning to basal values within 24 h (Yang et al., 2005), such events may be linked by common signaling and/or regulatory mechanisms, such as the restoration of muscle energy stores, predominantly glycogen.

The original “train-low” protocol advocated twice-a-day training sessions in which only the second exercise session was undertaken with low glycogen availability (Hansen et al., 2005). A direct consequence of implementing this strategy with athletes was that the maximal self-selected training intensity of the second session was substantially reduced when it was

commenced with low, compared to normal (or elevated) glycogen levels (Hulston et al., 2010, Yeo et al., 2008b). Such an outcome is counterintuitive for the preparation of competitive athletes where high-intensity workouts are a critical component of a periodized training program (Hawley and Morton, 2013). Against this background, a novel approach was formulated in which the duration of low carbohydrate (i.e., muscle and liver glycogen) availability was prolonged, thereby potentially enhancing and extending the time course of transcriptional activation of metabolic genes and their target proteins, while simultaneously conserving the training ‘impulse’ to the working muscles. This strategy was termed “train-high, sleep-low”. In this model, an athlete would commence a high-intensity training (HIT) session in the evening with high glycogen availability (i.e., “train-high”), then go to bed fasted (i.e., “sleep-low”), before undertaking a subsequent prolonged, submaximal training session the next morning before re-feeding. The main hypothesis was that delaying energy (i.e., carbohydrate) intake and extending the duration an individual was in a low glycogen state would enhance the acute responses of selected genes and proteins with putative roles in training adaptation, compared to when individuals followed sports nutrition guidelines (i.e., high post-exercise carbohydrate availability). A second hypothesis was that the same subset of genes and proteins measured in the early recovery phase after HIT would remain elevated after sleeping low, and demonstrate greater activation subsequent to performing a second submaximal training session the following morning.

### **3.2 Methods**

#### *Subjects*

Seven male competitive endurance trained cyclists with a history of >3 yr endurance training and who were riding an average of  $406 \pm 59 \text{ km}\cdot\text{wk}^{-1}$  (range 285-455  $\text{km}\cdot\text{wk}^{-1}$ , values mean  $\pm$

SD) in the 6 wk prior to commencement of the study, volunteered to participate in these trials. The subjects' age, body mass (BM), peak oxygen uptake ( $\dot{V}O_{2\text{peak}}$ ) and peak power output (PPO) (Hawley and Noakes, 1992) were  $29 \pm 5$  yr,  $76.9 \pm 9.1$  kg,  $67 \pm 4.0$  mL $\cdot$ kg $^{-1}\cdot$ min $^{-1}$  and  $422 \pm 39$  W. Prior to giving their written consent all subjects were informed of the possible risks of all procedures. The study was approved by the RMIT Human Research Ethics Committee.

### *Study overview*

Each subject completed two experimental trials in a randomised cross-over design. In each trial they performed two exercise bouts: the first bout (high-intensity training, HIT) was undertaken in the evening of the first day, and the second bout (prolonged, steady state ride; 120SS) on the morning of the second day. In one trial subjects consumed half of their total daily energy intake ( $8$  g $\cdot$ kg $^{-1}$  BM of CHO,  $1.5$  g $\cdot$ kg $^{-1}$  BM of protein and  $1.5$  g $\cdot$ kg $^{-1}$  BM of fat) throughout the day before undertaking the HIT session in the evening (1900-2000 h). They then remained fasted overnight (FAST). In the other trial, subjects ate half of the provided food ( $4$  g $\cdot$ kg $^{-1}$  BM of CHO,  $0.75$  g $\cdot$ kg $^{-1}$  BM of protein and  $0.75$  g $\cdot$ kg $^{-1}$  BM of fat) prior to the evening HIT session, and then consumed the remainder immediately after HIT (FED). Subjects remained in the laboratory overnight and completed 120SS commencing at 0700 h the following morning. One hour after the completion of 120SS all subjects consumed a standardised breakfast containing  $2$  g $\cdot$ kg $^{-1}$  BM CHO and remained in the laboratory until the completion of the trial at  $\sim 13:00$ . Skeletal muscle biopsies were obtained before and 2 h post HIT (day 1) and at rest, immediately post the 120min Steady State ride (120 SS) and after 4 h recovery (day 2) and analysed for selected markers of training adaptation.

### *Pretesting: Incremental cycle test*

Approximately 2 wk prior to commencement of their first experimental trial all subjects underwent an incremental cycling test to exhaustion on an electronically braked cycle ergometer (Lode Excalibur *Sport*, Groningen, The Netherlands) as previously described (Hawley and Noakes, 1992). During this maximal test subjects breathed through a Hans Rudolph two-way non-rebreathing valve and mouth piece attached to a calibrated online gas system (TrueOne 2400, Parvomedics, Utah, USA) interfaced to a computer that calculated the instantaneous rates of  $\text{O}_2$  consumption ( $\dot{V} \text{O}_2$ ),  $\text{CO}_2$  production ( $\dot{V} \text{CO}_2$ ), minute ventilation ( $\text{VE}_{\text{STPD}}$ ), and the respiratory exchange ratio (RER). Before each test, analysers were calibrated with commercially available gasses of known  $\text{O}_2$  and  $\text{CO}_2$  content.  $\dot{V} \text{O}_{2\text{peak}}$  was defined as the highest uptake a subject attained during any 30 s of the test while PPO was calculated from the last completed work rate plus the fraction of time spent in the final non-completed work rate as previously described (Hawley and Noakes, 1992). The maximal test and all experimental trials were conducted under standardised laboratory conditions (18-22°C, 40-50% relative humidity) and subjects were fan cooled during all exercise sessions. Each individual's PPO recorded during the incremental test was used to determine their prescribed cycling intensities (Watts) during the subsequent experimental trials.

### *Standardised Diet/Exercise control*

Subjects consumed a pre-packaged standardised diet for the 24 h period prior to commencing an experimental trial (Jeacocke and Burke, 2010). Dietary goals for this period were 8 g·kg<sup>-1</sup> BM CHO; 1.5 g·kg<sup>-1</sup> BM protein; 1.5 g·kg<sup>-1</sup> BM fat; for a total energy intake of ~220 kJ·kg<sup>-1</sup> BM for the 24 h period. Subjects were instructed to avoid any strenuous physical activity as well as alcohol and caffeine consumption for the 24 h prior to a trial. Subjects were provided

with all foods and drinks in portion controlled packages for consumption during the dietary control period and were given verbal and written instructions on how to follow the diet. Checklists were used to record each menu item as it was consumed and to note any deviations from the menu. Each subject's food checklists were checked and clarified for compliance to the standardisation protocols by the primary researcher.

### *Experimental Diet*

On the day of an experimental trial subjects were provided with all food and fluid to be consumed prior to reporting to the laboratory at ~ 17:00. Subjects received one of two isoenergetic diets (containing 8 g·kg<sup>-1</sup> BM CHO; 1.5 g·kg<sup>-1</sup> BM protein; 1.5 g·kg<sup>-1</sup> BM fat; for a total energy intake of ~220 kJ·kg<sup>-1</sup> BM) that only differed in the timing of consumption. During one trial (FASTED) food was portioned such that subjects consumed 6 g·kg<sup>-1</sup> BM CHO throughout the day with their final day's meal (2 g·kg<sup>-1</sup> BM CHO) being consumed at ~17:00 upon arrival at the laboratory. During the other trial (FED) food was portioned so that subjects consumed 2 g·kg<sup>-1</sup> BM CHO before 1700 h, a further 2 g·kg<sup>-1</sup> BM CHO meal upon arrival at the lab (1700 h) with the remainder of that day's intake (4 g·kg<sup>-1</sup> BM CHO) was consumed after the HIT session at ~20:00. On day 2, a breakfast containing 2 g·kg<sup>-1</sup> BM CHO was consumed 1 h after completion of the 120SS ride in both trials.

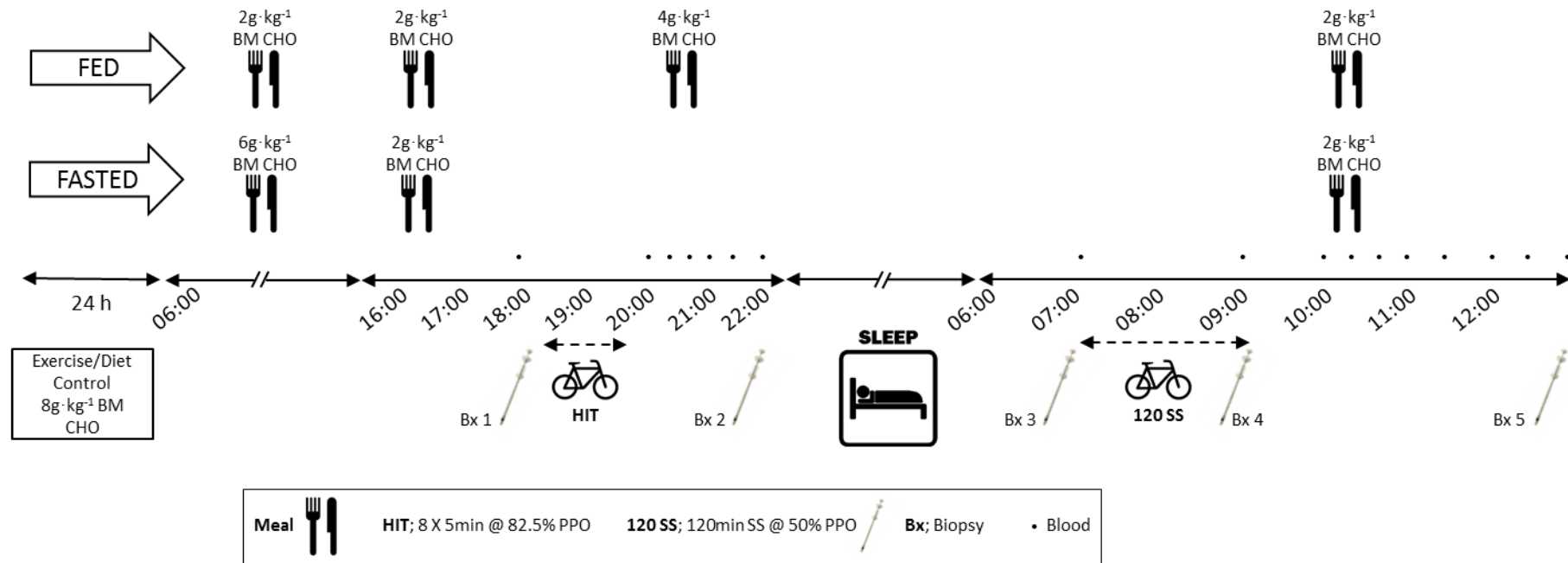
### *Blood and tissue collection and analysis*

Seventeen blood samples were collected during each trial with a total 9 mL of whole blood obtained at each sampling time point (Figure 1). Six mL of blood was collected in tubes containing EDTA. Twenty-five µL of blood was then immediately analysed for glucose



concentration (YSI, Yellow Springs, Ohio, USA) while the remaining sample was then immediately centrifuged at 4°C at 4000 rev·min<sup>-1</sup> (3040 x g) for 10 min with the resulting plasma transferred to 1.5 mL tubes and stored at -80 °C for subsequent analyses of plasma insulin and catecholamine concentrations. At each time point a further 3 mL of blood was collected in a tube containing EGTA which was then centrifuged and the resulting plasma frozen and stored (as described above) for later analyses of free fatty acid (FFA). Catecholamine concentrations were analysed using a commercially available enzyme immunoassay (Bi-CAT EIA 17-BCTHU-E02.1, ALPCO, Salem NH), while plasma insulin concentrations were determined via ELISA (80-INSHU-E01.1, E10.1, ALPCO, Salem NH). Plasma FFA concentrations were determined using an enzymatic colorimetric method (NEFAC code 279-75409, Wako, Tokyo Japan).

A total of five biopsies were collected during each of the experimental trials from the *vastus lateralis* using a 5 mm Bergström needle adapted for manual suction. Samples were immediately washed in 0.9 % saline solution then snap frozen in liquid nitrogen and stored at -80°C until later analysis. The sampling points were before and 2 h after HIT (day 1) and then at rest, immediately post 120SS and after 4 h recovery (day 2). Biopsies were subsequently analysed for gene and protein markers of training adaptation (described subsequently).



**Figure 3.1:** Experimental design. FED (A total of 4g CHO kg<sup>-1</sup> BM prior to HIT and 4g CHO kg<sup>-1</sup> BM post HIT), FASTED (A total of 8g CHO kg<sup>-1</sup> BM prior to HIT and remained fasted during sleep and throughout 120SS).

### *High Intensity Interval Session (HIT)*

On the evening of the first day of an experimental trial subjects completed a HIT session on a Velotron cycle ergometer (Racermate, Seattle, WA, USA). After a standardised warm up (10 min at 60 % PPO) subjects undertook HIT, which consisted of 8 x 5 min work bouts at 82.5% of individual PPO with 1 min active recovery (100 W) between work bouts. This protocol was chosen as the physiological demands have previously been characterised and in well-trained athletes, the session reduces muscle glycogen by ~ 50% of starting values (Stepto et al., 2001). During HIT, ratings of perceived exertion (RPE) were recorded at the end of each work bout while heart rate was averaged for each 5 min repetition.

### *120min Steady State ride (120SS)*

On the morning of the second day of each trial subjects completed a 120min steady state cycling bout (120SS) on the same ergometer as used for HIT. During this ride subjects cycled at ~60% of  $\dot{V}O_{2\text{peak}}$ . The respiratory exchange ratio (RER) was recorded as a 5min average commencing at 10, 45, 80, 115 min, while heart rate and RPE were recorded at the end of each 5 min collection point. Whole body rates of CHO and fat oxidation (g/min) were calculated from the respiratory data collected during the 120SS ride. The calculations were made from  $\dot{V}CO_2$  and  $\dot{V}O_2$  measurements, assuming a non-protein RER value, according to the following equation (Peronnet and Massicotte, 1991).

$$\text{CHO oxidation} = 4.585 \dot{V}CO_2 - 3.226 \dot{V}O_2$$

$$\text{Fat oxidation} = 1.695 \dot{V}O_2 - 1.701 \dot{V}CO_2$$

### *Fluid intake*

During the first experimental trial (including the 24 h standardized dietary control) subjects were allowed water *ad libitum*. The volume of fluid consumed was recorded and then replicated during the subsequent trial.

### *Muscle glycogen concentration*

Muscle glycogen concentration was analysed as previously described (Churchley et al., 2007). In brief, approximately 10-15mg of muscle was freeze dried and powdered, with all visible blood and connective tissue removed under magnification. The freeze-dried muscle sample was then extracted and glycogen concentration determined via enzymatic analyses.

### *RNA Extraction and Quantification*

Approximately 20 mg of skeletal muscle was homogenised in TRIzol and chloroform added to form an aqueous RNA phase. This RNA phase was then precipitated by mixing with isopropanol alcohol and the resulting pellet was washed and re-suspended in 50 µL of RNase-free water. Extracted RNA was quantified using a QUANT-iT analyser kit (Invitrogen, Melbourne, Australia, Cat No Q32852) and on a NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA) by measuring absorbance at 260 nm and 280 nm with a 260/280 ratio of ~ 1.88 recorded for all samples.

### *Reverse Transcription and Real-Time PCR*

First-strand complementary DNA (cDNA) synthesis was performed using commercially available TaqMan Reverse Transcription Reagents (Invitrogen, Melbourne, Australia) in a final reaction volume of 20  $\mu$ L. All RNA and negative control samples were reverse transcribed to cDNA in a single run from the same reverse transcription master mix. Serial dilutions of a template RNA (AMBION; Cat No AM7982) was included to ensure efficiency of reverse transcription and for calculation of a standard curve for real-time quantitative polymerase chain reaction (RT-PCR). Quantification (in duplicate) was performed using a Rotor-Gene 3000 Centrifugal Real-Time Cycler (Corbett Research, Mortlake, Australia). Taqman-FAM-labelled primer/probes for PGC-1 $\alpha$  (Cat No. Hs01016719), Tfam (Cat No. Hs00273372\_s1), COXIV (Cat No. Hs01872840\_s1), PPAR  $\delta$  (Cat No. Hs04187066\_g1), CD36 (Cat No. Hs01567185\_m1), FABP (Cat No. Hs01086177\_m1), PDK4 (Cat No. Hs01037712\_m1), and GLUT-4 (Cat No. Hs00168966\_m1) were used in a final reaction volume of 20  $\mu$ L. PCR treatments were 2 min at 50 °C for UNG activation, 10 min at 95 °C then 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat No Hs Hs99999905) was used as a housekeeping gene and expression was not different at any time point or between treatments (data not shown). The relative amounts of mRNAs were calculated using the relative quantification ( $\Delta\Delta$ CT) method (Livak and Schmittgen, 2001).

### *Western Blot Analysis*

Muscle samples (~15 mg) were homogenized in ice-cold buffer containing 50 mM of Tris–HCl, pH 7.5, 1 mM of EDTA, 1 mM of EGTA, 10% glycerol, 1% Triton X-100, 50 mM of NaF, 5 mM of sodium pyrophosphate, 1 mM of DTT, 10  $\mu$ g·mL<sup>-1</sup> of trypsin inhibitor, 2

$\mu\text{g}\cdot\text{mL}^{-1}$  of aprotinin, 1 mM of benzamidine, and 1 mM PMSF using a motorised pellet pestle (Sigma-Aldrich, St. Louis, MO) with 5 s pulses. The lysate was kept on ice at all times and was then centrifuged at 12,000 x g for 20 min at 4°C. The supernatant was transferred to a sterile tube and was subsequently aliquoted for determination of protein concentration using a BCA protein assay (Pierce, Rockford, IL). The supernatant was subsequently resuspended in Laemelli sample buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes and incubated with primary antibody (1:1,000) overnight at 4°C and secondary antibody (1:2,000), and proteins detected via chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology, Rockford, IL) and quantified by densitometry (Chemidoc, BioRad, Gladesville, Australia). All sample (50  $\mu\text{g}$ ) time points for each subject were run on the same gel. Polyclonal anti-phospho AMPK $\alpha^{\text{Thr172}}$  (no. 2531), -ACC $^{\text{Ser79}}$  (no. 3661P), -HSL $^{\text{Ser660}}$  (no. 4126S), monoclonal anti-phospho p38MAPK $^{\text{Thr180/Tyr182}}$  (no. 4511) and monoclonal total ATGL (no. 2439S) and CPT1A (no. 12252S) were purchased from Cell Signaling Technology (Danvers, USA). Data are expressed relative to  $\alpha$ -tubublin (no. 3873, Cell Signaling Technology, Danvers, USA) in arbitrary units.

### *Statistical Analysis*

Respiratory, physiological, blood, muscle glycogen and PCR data were analysed using SPSS software package (version 21). Western blot data were analysed using Sigma Stat (version 3.1). All data were checked for sphericity using Mauchly's Test and normality using Kolomogorov-Smirnov tests. Due to identified violations, data for mRNA were natural log transformed before further analyses. To compare the responses during the experimental trials data were analysed using two-way Analyses of Variance (Trial by Time) with repeated

measures ( $\alpha = 0.05$ ). Least Significant Difference and paired 't' tests were used post-hoc. Results and statistics represent seven subjects unless otherwise indicated. All values are expressed as means  $\pm$  SD unless otherwise indicated whereas protein and mRNA data are expressed as means  $\pm$  percentile range where  $n = 7$ , 16-83%;  $n = 6$ , 20-80%;  $n = 5$ , 25-75%;  $n = 4$ , 33-66%).

### 3.3 Results

#### *High Intensity Intervals (HIT)*

##### *Training Responses*

During HIT two subjects were unable to complete the 8 x 5 min intervals at the prescribed intensity during their first trial (subject 4 at interval 4 and subject 8 at interval 5). As such, intensity was reduced by 10 W for subsequent intervals to allow the subjects to complete the remaining work bouts and an identical set of work bouts repeated for their second trial. Accordingly, there were no differences in average power output sustained for all work bouts between trials for any subject (FED and FASTED,  $346\text{W} \pm 31\text{W}$ ). Heart rate and RER response are shown in Table 3.1. There were no differences in average RPE (FED  $16.2 \pm 1.4$  vs FASTED  $15.9 \pm 1.5$ ) or average heart rate between trials (FED  $171 \pm 10$  bpm vs FASTED  $173 \pm 11$  bpm).

##### *Submaximal cycling bout (120SS)*

During the 120 SS ride on the morning of day 2, RER was lower in FASTED at 10-15, 45-50 and 115-120min time points during FAST compared with FED (Table 3.1). During both trials there was a main effect of time ( $P < 0.01$ ) where there was a steady decline in RER during the

ride. There were no differences in HR between trials at any time point. Heart rate tended to increase throughout 120SS although there was no main effect of time for trials ( $P = 0.056$ ). In the FED trial only, HR was statistically higher at 115-120 min when compared to 10-15 ( $P = 0.01$ ). There were no differences in RPE between trials at any point. Similarly, there was no effect of time on RPE during trials.



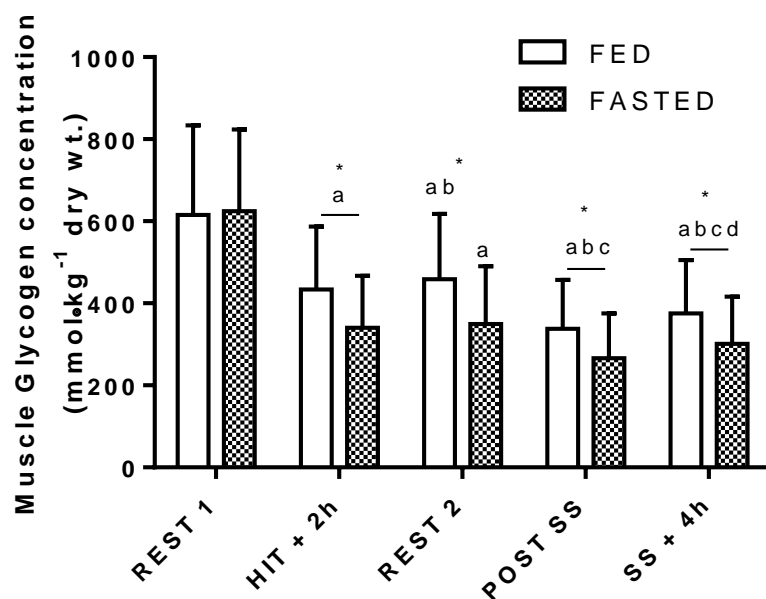
**Table 3.1:** Physiological and respiratory response to HIT and 120SS.

HIT (8 x 5min Intervals at 82.5% Peak Power Output)										
		Int 1	Int 2	Int 3	Int 4	Int 5	Int 6	Int 7	Int 8	Mean
<b>RPE</b>	FED	13.9 ± 1.2	15.4 ± 1.6	15.5 ± 1.0	16.4 ± 1.0	17.0 ± 1.3	17.1 ± 1.3	17.0 ± 1.7	17.0 ± 1.7	<b>16.2 ± 1.4</b>
	FASTED	13.6 ± 1.3	14.8 ± 0.8	15.4 ± 0.9	16.4 ± 2.3	15.6 ± 1.9	16.8 ± 1.8	17.2 ± 1.6	17.4 ± 1.1	<b>15.9 ± 1.5</b>
<b>Heart Rate</b>	FED	166 ± 10.4	168 ± 11.1	171 ± 11.7	171 ± 9.8	171 ± 9.7	173 ± 9.2	173 ± 9.3	173 ± 10.6	<b>171 ± 10.2</b>
	FASTED	168 ± 11.7	171 ± 12.8	173 ± 12.7	173 ± 11.7	173 ± 11.2	175 ± 10.7	175 ± 10.8	175 ± 12.2	<b>173 ± 11.7</b>
120 Steady State										
		10-15min	45-50min	80-85min	115-120min	Mean				
<b>RER</b>	FED	0.88 ± 0.03	0.86 ± 0.03 <sup>f</sup>	0.84 ± 0.03 <sup>fg</sup>	0.84 ± 0.03 <sup>fg</sup>	<b>0.85 ± 0.03</b>				
	FASTED	0.85 ± 0.04 *	0.82 ± 0.04 <sup>*f</sup>	0.82 ± 0.04 <sup>fg</sup>	0.80 ± 0.03 <sup>*fgh</sup>	<b>0.82 ± 0.04*</b>				
<b>RPE</b>	FED	10.6 ± 1.0	10.4 ± 1.1	10.7 ± 1.1	10.9 ± 0.7	<b>10.6 ± 0.20</b>				
	FASTED	10.2 ± 0.8	10.4 ± 1.3	10.6 ± 1.1	10.8 ± 0.8	<b>10.5 ± 0.24</b>				
<b>Heart Rate</b>	FED	133 ± 7.5	136 ± 8.1	136 ± 8.9	138 ± 7.6 <sup>f</sup>	<b>136 ± 0.65</b>				
	FASTED	135 ± 7.9	136 ± 7.3	135 ± 6.8	137 ± 6.4	<b>136 ± 0.62</b>				

Values are mean ± SD, P < 0.05; (f) different to 10-15min, (g) different to 45-50min, (h) different to 80-85min. (\*) different to FED.

### *Muscle Glycogen Concentration (Figure 3.2)*

Prior to the HIT session, there were no differences in muscle glycogen concentrations for the two experimental conditions. Two hours post-HIT muscle glycogen concentration was reduced by ~45% in the FASTED trial and by ~30% in FED compared to REST 1. Resting muscle glycogen concentrations on the morning of day 2 (REST 2) remained lower than REST 1 ( $P < 0.05$ ) and consequently the 120SS bout was commenced with significantly lower glycogen concentration in the FASTED trial ( $349 \pm 141 \text{ mmol} \cdot \text{kg}^{-1} \text{ dry wt}$ ) compared to FED ( $459 \pm 159 \text{ mmol} \cdot \text{kg}^{-1} \text{ dry wt}$ ) ( $P < 0.01$ ). The 120SS bout further reduced glycogen concentrations in both trials by approximately 25% with a greater absolute reduction in the FED ( $121 \pm 42 \text{ mmol} \cdot \text{kg}^{-1} \text{ dry wt}$ ) versus FASTED ( $83 \pm 39 \text{ mmol} \cdot \text{kg}^{-1} \text{ dry wt}$ ) trial. The meal consumed 1 h post the 120SS ride elevated glycogen concentrations 3 h post feeding ( $P < 0.001$ ) to a similar extent in both trials ( $\sim 25 \text{ mmol} \cdot \text{kg}^{-1} \text{ dry wt}$ ). Additionally, muscle glycogen concentrations in the FASTED trial remained below that of the FED trial ( $P < 0.01$ ).



**Figure 3.2:** Skeletal muscle glycogen concentration. (\*) difference between trials (a) different to REST 1, (b) different to HIT +2h, (c) different to REST 2, (d) different to POST SS, Values are mean  $\pm$  SD,  $P < 0.05$

### *Blood glucose*

Blood glucose concentrations are displayed in Figure 3.3A. At REST 1, immediately before the HIT exercise bout and 2 h post the standardized meal blood glucose concentrations were slightly higher in the FASTED trial compared to the FED trial ( $P < 0.05$ ), reflecting the greater overall CHO intake in the FASTED trial to this point in the day. Immediately post HIT blood glucose concentrations did not differ between the trials. The meal consumed immediately post-HIT in the FED trial increased blood glucose concentrations with levels peaking at HIT+30 then gradually declining, but still being slightly elevated above FASTED at HIT+90. Immediately prior to 120SS ride on the morning of day 2 (REST 2), immediately after the ride and then throughout recovery, blood glucose concentrations in both trials displayed similar profiles: initially increasing as a consequence of the meal that was consumed 1 h post-the 120min SS ride and then declining until the end of the trials. In both trials blood glucose concentrations peaked 40 min after the meal (120SS+100) and declined to REST 1 concentrations over the subsequent 3 h, with a small difference between trials 150 min post-meal (120SS+210 min), at which point blood glucose in the FASTED trial was slightly higher than that in the FED trial.

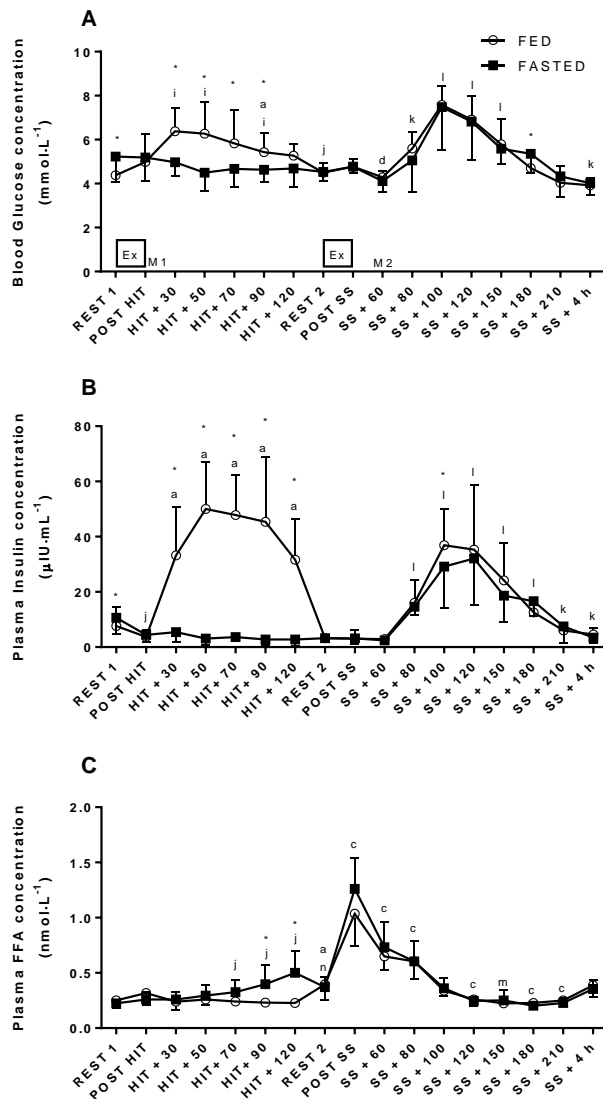
### *Insulin*

Plasma insulin concentrations are displayed in Figure 3.3B. At REST 1, plasma insulin was higher in the FASTED trial compared to the FED trial, which reflected the higher blood glucose concentration in the FASTED trial and is likely due to the larger daily CHO intake in the FASTED trial prior to this point. The meal consumed in the FED trial immediately post-HIT significantly elevated plasma insulin values compared to POST HIT concentrations, as well the FASTED trial for all time points until the final blood collection point of the day at

HIT+120. The following morning at REST2 and immediately post the 120SS bout insulin concentrations were similar between trials and near resting levels. Following the post 120SS meal (eaten 60 min post exercise in both trials), plasma insulin concentrations peaked 40 min post ingestion in the FED trial and 60 min post ingestion in the FASTED trial. Plasma insulin concentrations then declined in a similar manner in both trials returning to REST 1 concentrations within 3 h post ingestion.

#### *Plasma FFA*

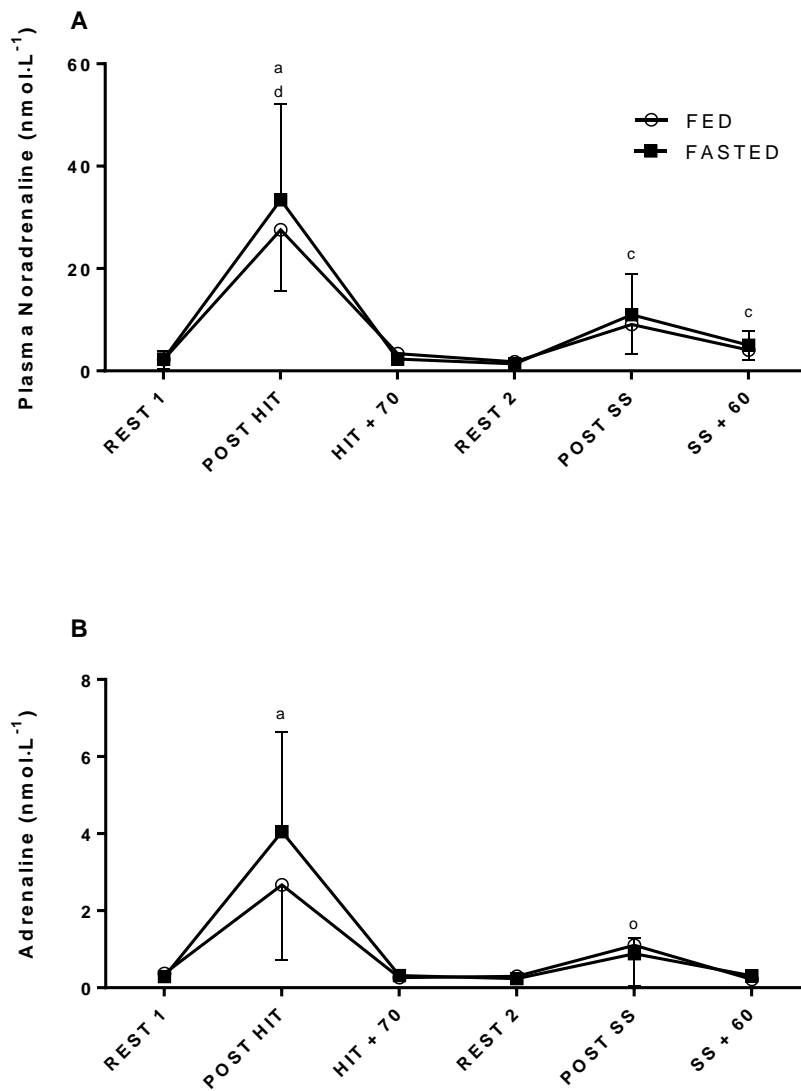
Plasma FFA concentrations are displayed in Figure 3.3C. There were no differences between trials at REST 1, or immediately post HIT. In the FASTED trial plasma FFA concentrations continued to increase above REST 1 and compared to the FED trial with levels becoming elevated at time points 90 and 120 min post-HIT. Resting plasma FFA concentrations after the overnight sleep had declined from their previously elevated concentrations in the FASTED trial, but were still above REST 1 concentrations. There were no differences in plasma FFA levels between trials at this time, The 120min SS ride was associated with elevated plasma FFAs in both trials ( $P < 0.01$ ) with no difference observed between trials ( $P = 0.179$ ). Following breakfast, plasma FFA concentrations continued to decline in both trials, returning to REST 2 concentrations 40 min post ingestion (120SS+100).



**Figure 3.3:** Blood and plasma responses (A) Blood glucose concentration, (B) Plasma insulin concentration, (C) Plasma FFA concentration. \* difference between trials (a) different to REST 1, (c) different to REST 2, (d) different to POST SS, (i) different to REST 1 in FED only (j) different to REST 1 in FASTED only, (k) different to SS + 60 in FED only, (l) different to SS + 60, (m) different to REST 2 in FED only, (Ex) Exercise, (M1) Meal in FED; 4 g.kg<sup>-1</sup> BM CHO, (M2) Meal in both trials; 2 g.kg<sup>-1</sup> BM CHO, Values are mean  $\pm$  SD,  $P < 0.05$ .

### *Catecholamines*

Plasma adrenaline and noradrenaline concentrations are displayed in Figure 3.4. Concentrations were not different between trials at REST 1 or at any other time point throughout the trials. Both catecholamines were significantly elevated post-HIT ( $P < 0.01$ ) but had returned to REST 1 concentrations within 60 min of completing HIT. After the 120SS ride on day 2, plasma noradrenaline was elevated in both trials, but to a lesser magnitude than following the HIT session, reflecting the lesser intensity of the 120SS ride ( $P = 0.02$ ). However, the plasma noradrenaline concentrations were still slightly above REST 2 concentrations 60 min post exercise ( $P = 0.02$ ). Plasma adrenaline concentrations displayed a similar profile to the noradrenaline response and immediately post 120SS were above REST 2 concentrations, but this only reached statistical significance in the FASTED trial ( $P = 0.005$ ), and had returned to REST 2 concentrations within 60 min post-exercise in both trials.

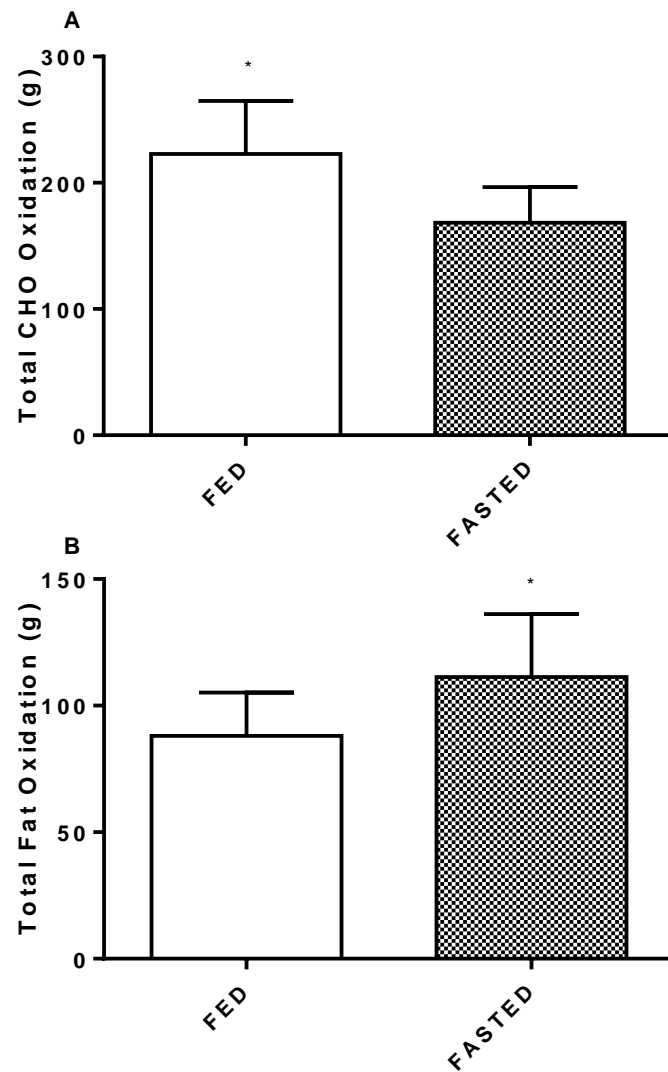


**Figure 3.4:** Plasma Catecholamine concentrations, (A) Noradrenaline concentration, (B) Adrenaline concentration, (a) different to REST 1, (c) different to REST 2, (d) different POST SS, (o) Adrenaline different to REST 2 in FASTED only, Values are mean  $\pm$  percentile range,  $P < 0.05$ .



### *Substrate utilisation during 120SS*

Figure 3.5 shows the estimated total oxidation of CHO and fat ( $\text{g} \cdot 120 \text{ min}^{-1}$ ) during the 120SS cycling bout. Total CHO oxidation was greater in FED ( $223 \pm 42 \text{ g}$ ) than FASTED ( $168 \pm 28 \text{ g}$ ) ( $P = 0.01$ ), while total fat oxidation was greater in FASTED ( $111 \pm 25 \text{ g}$ ) compared to FED ( $88 \pm 17 \text{ g}$ ) ( $P = 0.01$ ).

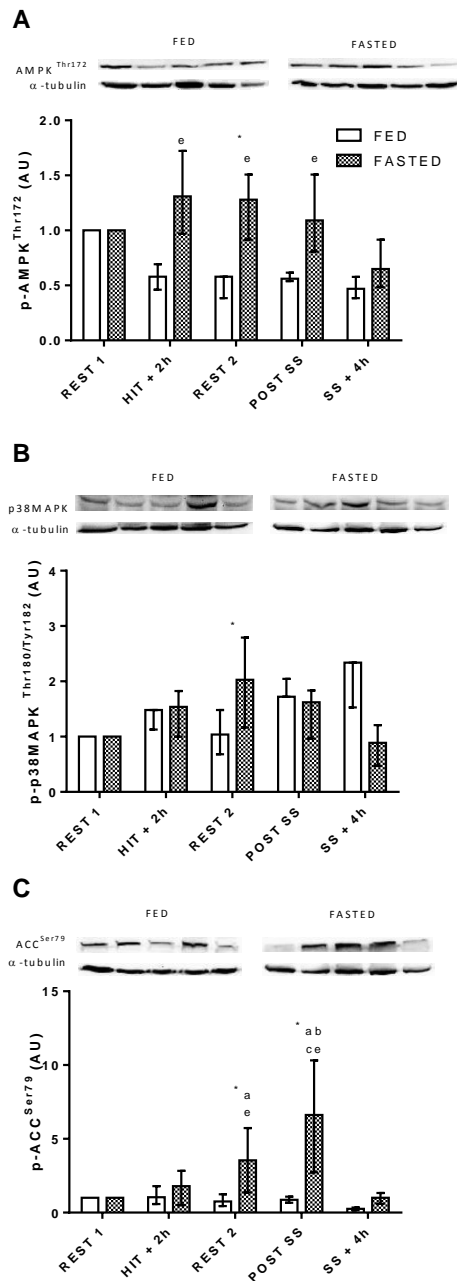


**Figure 3.5:** Total carbohydrate and fat oxidation during the 120SS bout. (A) Total carbohydrate oxidation, (B) Total fat oxidation. (\*) difference between trials, Values are mean  $\pm$  SD,  $P < 0.05$ .

## *Protein data*

### *Signaling proteins (Figure 3.6)*

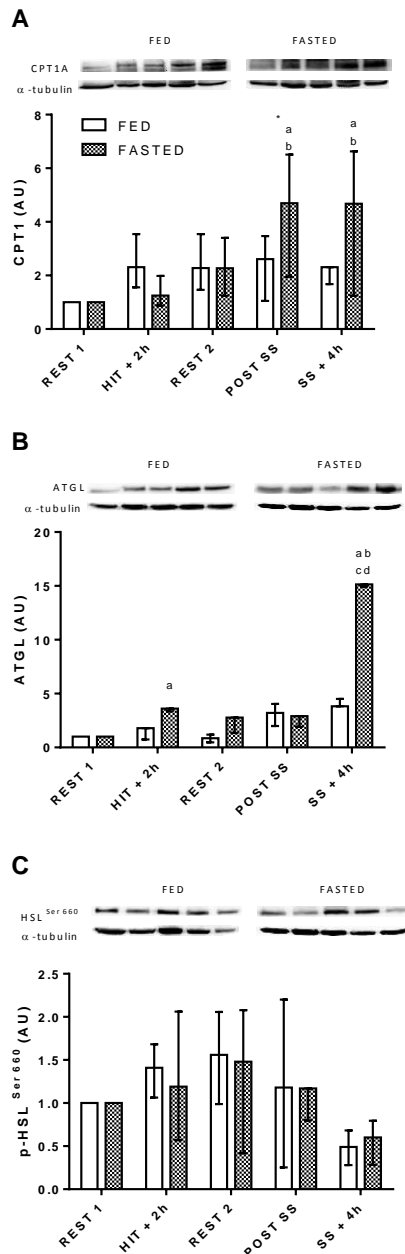
Baseline values for p-AMPK<sup>Thr172</sup>, p-p38MAPK<sup>Thr180/Tyr182</sup> and p-ACC<sup>Ser79</sup> were similar at rest on day 1. At 2 h post-HIT p-AMPK<sup>Thr172</sup> tended to be higher in FASTED compared to the FED trial (P = 0.058). The following morning (REST 2), the phosphorylation of AMPK<sup>Thr172</sup>, p38MAPK<sup>Thr180/Tyr182</sup> and ACC<sup>Ser79</sup> was greater in the FASTED compared to the FED trial, while p-ACC<sup>Ser79</sup> was also elevated compared to REST 1 (P <0.05). The 120min SS bout (POST SS) did not increase p-AMPK<sup>Thr172</sup> or p-p38MAPK<sup>Thr180/Tyr182</sup> above REST 2. However, p-ACC<sup>Ser79</sup> further increased in FASTED at POST SS (P <0.05) but had returned to resting values by SS+4h p-AMPK<sup>Thr172</sup> showed a similar pattern.



**Figure 3.6:** Signaling proteins. (A) Phosphorylation of 5' adenosine monophosphate-activated protein kinase (p-AMPK<sup>Thr172</sup>), (B) Phosphorylation of p38 mitogen-activated protein kinase (p-p38MAPK<sup>Thr180/Tyr182</sup>), (C) Phosphorylation Acetyl-CoA carboxylase (p-ACC<sup>Ser79</sup>). (\*) difference between trials, (a) different to REST 1, (b) different to HIT + 2h, (c) different to REST 2, (e) different to SS + 4h; for all proteins at POST SS n = 6, Values are normalised to REST 1 and are expressed as means  $\pm$  percentile range,  $P < 0.05$ .

*Lipolysis and fat transport proteins (Figure 3.7)*

There were no differences between the two trials for CPT1, ATGL or p-HSL<sup>Ser 660</sup> at rest on day 1. At HIT +2h ATGL in the FASTED trial and was elevated compared to REST 1 ( $P < 0.05$ ). At POST SS CPT1 was higher in FASTED compared to FED ( $P < 0.01$ ) and was elevated compared to both time points from the previous day ( $P < 0.01$ ) and CPT1 remained elevated above these levels at SS +4h in the FASTED trial ( $P < 0.01$ ). ATGL was significantly higher at SS +4h in FASTED compared to all prior time points ( $P < 0.05$ ) but despite being substantially elevated was not significantly different to FED ( $P = 0.06$ ), possibly due to a lack of statistical power ( $n = 4$ ).

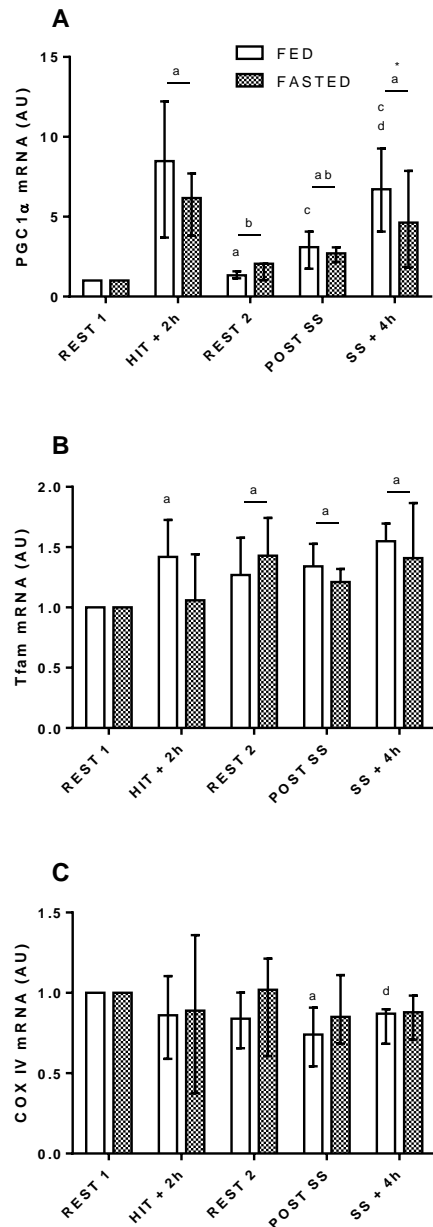


**Figure 3.7:** Lipolysis and fat transport proteins. (A) Carnitine palmitoyltransferase I (CPT1), (B) Adipose triglyceride lipase (ATGL), (C) Phosphorylation of Hormone sensitive lipase (p-HSL<sup>Ser 660</sup>), (a) different to REST 1, (b) different to HIT +2h, (c) different to REST 2, (d) different to POST SS; at all time points CPT1 n = 6, ATGL n=4, HSL n=5; Values are normalised to REST 1 and are expressed as means  $\pm$  percentile range,  $P < 0.05$ .

## *mRNA data*

### *Mitochondrial genes (Figure 3.8)*

There were no differences between trials for any of PGC1 $\alpha$ , Tfam or COX VI mRNA at rest. After HIT PGC1 $\alpha$  was increased in both trials ( $P < 0.05$ , ~6 fold-change). Tfam was increased in the FED trial only ( $P < 0.01$ ). At REST 2 PGC1 $\alpha$  had declined in both trials but remained slightly elevated in FED compared to REST 1 ( $P < 0.05$ ) while Tfam was still elevated in FED and increased in FASTED compared to REST 1. At POST SS PGC1 $\alpha$  and Tfam remained elevated compared to REST 1. Further increases in response to 120SS were only evident for PGC1 $\alpha$  in the FED trial, which also further increased at SS +4h reaching statistically higher levels compared to FASTED. COX IV declined below REST 1 values at POST SS then slightly increased again at SS +4h.

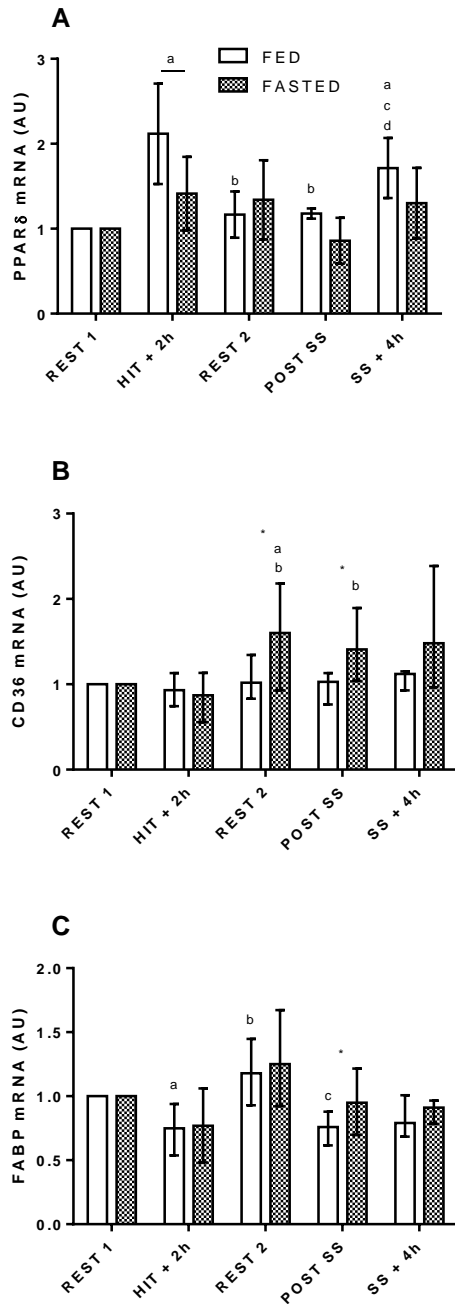


**Figure 3.8:** Mitochondrial genes, (A) Peroxisome proliferator-activated receptor gamma co-activator 1- $\alpha$  (PGC-1 $\alpha$ ), (B) Mitochondrial transcription factor A (Tfam), (C) Cytochrome c oxidase subunit IV (COX IV), \* difference between trials, (a) different to REST 1, (b) different to HIT +2h, (c) different to REST 2, (d) different to POST SS; COX IV at SS +4h n=6, Values are normalised to REST 1 and are expressed as means  $\pm$  percentile range,  $P < 0.05$ .



*Lipolysis and fat transport genes (Figure 3.9)*

There were no differences between trials at REST 1 for PPAR $\delta$ , CD36 or FABP. At HIT +2h mRNA was elevated for PPAR $\delta$  in both trials (FED ~2 fold,  $P=0.002$  and FASTED ~1.5 fold,  $P=0.038$ ). However, values for both trials had returned to pre-HIT levels by REST 2. CD36 in the FASTED trial increased overnight ( $P<0.05$ ) and was higher compared to FED ( $P=0.02$ ). FABP tended to increase overnight in both trials but only reached significance in FED compared to HIT +2h ( $P<0.05$ ). After the 120SS bout CD36 remained elevated in FASTED compared to FED while FABP tended to decline in both trials with a greater reduction occurring in FED. At SS +4h PPAR $\delta$  tended to increase in both trials with only FED reaching higher than previous values.

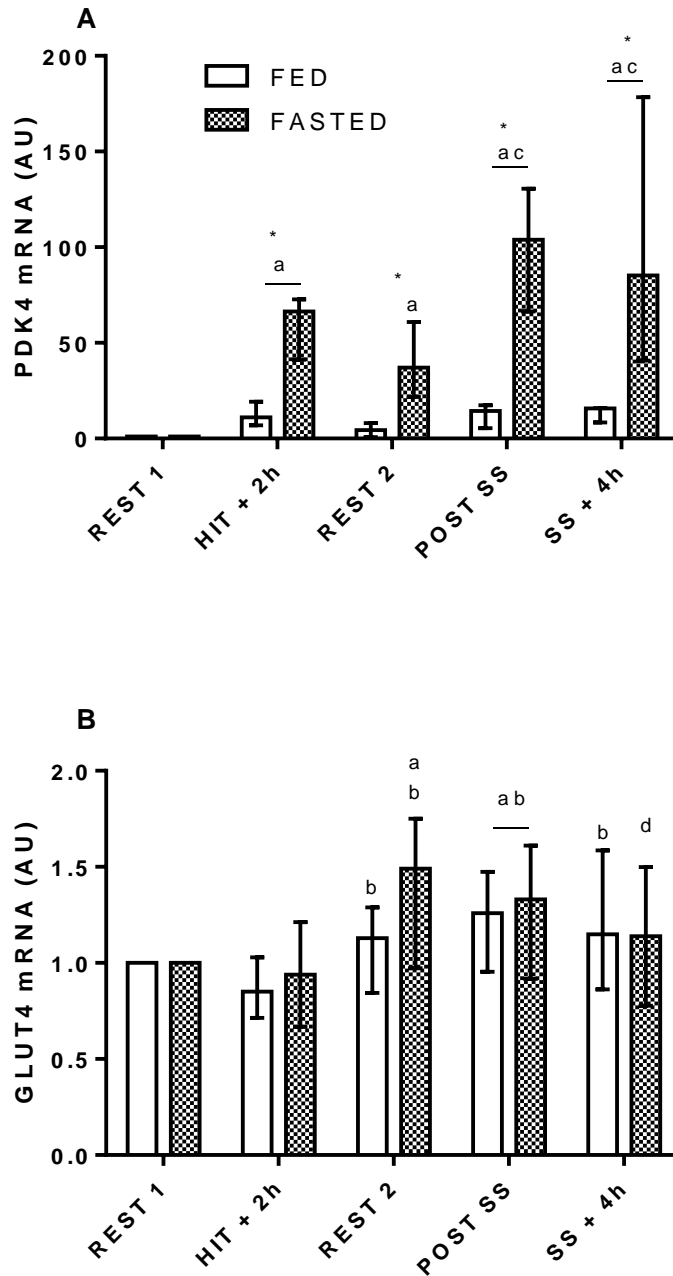


**Figure 3.9:** Lipolysis and fat transport genes, (A) Peroxisome proliferator-activated receptor delta (PPAR $\delta$ ), (B) Cluster of differentiation 36 (CD36), (C) Fatty acid binding protein (FABP). \* difference between trials, (a) different to REST 1, (b) different to HIT +2h, (c) different to REST 2; CD36 and FABP at SS +4h n=6, Values are normalised to REST 1 and are expressed as means  $\pm$  percentile range,  $P < 0.05$ .

*CHO oxidation genes (Figure 3.10)*

There were no differences between trials at REST 1 for either of PDK4 or GLUT4 mRNA.

At HIT +2 PDK4 was elevated in both trials ( $P < 0.01$ ), however was elevated to a greater extent in FASTED (~65 fold) compared to FED (~10 fold) ( $P = 0.03$ ). Overnight PDK 4 remained elevated compared to FED ( $P = 0.02$ ) and REST 1 ( $P < 0.01$ ) in FASTED only, while GLUT 4 increased in both trials compared to HIT +2 and remained elevated until POST SS. ( $P < 0.05$ ) The 120SS bout evoked further increases in PDK4 in both trials, which persisted until the final time point 4 h post 120SS ( $P < 0.05$ ) however, FASTED remained higher than FED ( $P < 0.05$ )



**Figure 3.10:** CHO oxidation genes. (A) Pyruvate dehydrogenase kinase 4 (PDK4), (B) Glucose transporter 4 (GLUT4), (\*) difference between trials, (a) different to REST 1, (b) different to HIT +2h, (c) different to REST 2, (d) different to POST SS. Values are normalised to REST 1 and are expressed as means  $\pm$  percentile range,  $P < 0.05$ .

### 3.4 Discussion

The current study determined selected cellular and whole-body responses to a novel exercise-nutrient intervention termed ‘train-high, sleep-low.’ The timing of nutrient intake was periodised such that athletes performed an evening bout of intense interval training with high-glycogen availability (‘train-high’) then restricted energy (i.e., carbohydrate) intake so that they slept with low glycogen availability (‘sleep-low’). The effects of this acute exercise-nutrient strategy on cellular markers of training adaptation and/or whole-body substrate metabolism during immediate recovery from interval training, as well as responses during and after a bout of prolonged, submaximal cycling, undertaken the following morning were examined. The main findings were that when feeding was withheld overnight and subjects slept with reduced energy availability, AMPK<sup>Thr172</sup>, p38MAPK<sup>Thr180/Tyr182</sup> and p-ACC<sup>Ser79</sup> were up-regulated to a greater extent the following morning, compared to when subjects were fed a high CHO meal early in recovery. It was also observed that when a second prolonged, steady-state training session was commenced after ‘sleeping low’, the abundance of selected genes and phosphorylation status of several signaling proteins with putative roles in lipid oxidation and transport were higher, compared to when a post-exercise meal was consumed, and glycogen availability was partially restored.

One of the main goals of this study was to circumvent the previously observed impairment in maximal self-selected training intensity when HIT is commenced with reduced muscle glycogen content (Yeo et al., 2008b, Hulston et al., 2010). Under such conditions, average power output is reduced by ~8% (Yeo et al., 2008b, Hulston et al., 2010) and even when caffeine is ingested to offset this decline, power output is still reduced by ~4% (Lane et al., 2013a) (Chapter 2). By undertaking HIT in the evening and then withholding feeding

throughout the night, athletes were able to complete the intense interval-training session and still ‘train-low’ the following morning without compromising the total ‘training impulse’ to the working muscles.

In the present study HIT reduced glycogen content by ~46% ( $280 \text{ mmol} \cdot \text{kg}^{-1}$  dry wt) which is of a similar magnitude to that reported in previous investigations using the same protocol and athletes of comparable training status (Stephens et al., 2001, Yeo et al., 2010). In comparison our observed mean reduction of ~46% and  $280 \text{ mmol} \cdot \text{kg}^{-1}$  dry wt is comparable to Stephens et al., (2001) who reported a  $250 \text{ mmol} \cdot \text{kg}^{-1}$  dry wt reduction (51%) in a similarly trained cohort who undertook an identical HIT bout. However, the pre-exercise glycogen content of the well-trained athletes in the current study was somewhat higher than previously observed ( $\sim 600 \text{ mmol} \cdot \text{kg}^{-1}$  dry wt; range  $\sim 400$  to  $\sim 900 \text{ mmol} \cdot \text{kg}^{-1}$  dry wt), and while the relative utilisation ( $\sim 50\%$ ) was in line with earlier work (Yeo et al., 2008b), in absolute terms there was still a substantial amount of glycogen left in the muscle after the HIT session ( $\sim 360 \text{ mmol} \cdot \text{kg}^{-1}$  dry wt; Figure 2). As such, athletes slept with reduced but not low muscle glycogen levels, while concomitantly commencing the next morning’s training session with higher than anticipated glycogen availability. Of note is that the glycogen content attained in the current study after the HIT session is certainly higher than concentrations reported previously by others who subsequently observed significant up-regulation of several training-induced signaling responses (Psilander et al., 2012, Bartlett et al., 2013, Morton et al., 2009, Pilegaard et al., 2002, Yeo et al., 2010).

Notwithstanding such differences in exercise-induced glycogen utilisation among studies, significant increases in PGC1 $\alpha$  mRNA abundance several hours after HIT (Figure 8 ) were

observed, which is in agreement with other investigations that have used a variety of glycogen-depleting protocols (Mathai et al., 2008, Pilegaard et al., 2003, Gibala et al., 2009, Cochran et al., 2010). PDK4 mRNA abundance was elevated compared to rest at 2 h post-HIT in both trials (Figure 10). However, the consumption of a high carbohydrate meal immediately after HIT blunted the rise in PDK4 mRNA such that levels in the FASTED trial were ~6 fold greater than in the FED trial, with differences between trials persisting at all subsequent time points.

The effects of ‘sleeping-low’ or ‘sleeping-high’ can be observed by examining markers of training adaptation/substrate availability in the resting tissue samples obtained on the morning of day two of the experiment. After withholding energy intake overnight, the phosphorylation state of AMPK<sup>Thr172</sup>, p38MAPK<sup>Thr180/Tyr182</sup> and p-ACC<sup>Ser79</sup> were elevated to a greater extent in the FASTED versus FED trial (Figure 6). Yet despite up-regulation of these signaling markers, both the protein content and mRNA abundance of several downstream targets (including Tfam and COXIV; Figure 8, and PPAR $\delta$ ; 9) did not follow the same temporal pattern. For example, compared to 2 h post HIT the prior evening, PGC1 $\alpha$  mRNA content had declined the morning of day 2 (some 10 h later) irrespective of whether or not a high carbohydrate meal was consumed. Pilegaard et al., (2005) have previously observed that when untrained, but physically active male subjects were fed isoenergetic meals containing either low or high carbohydrate content during recovery from exercise (75 min of cycling at 75%  $\dot{V} O_{2max}$ ), PGC1 $\alpha$  mRNA content was elevated to a similar magnitude under both conditions after 5 h of passive recovery. However, in contrast to the findings of the current study, these workers (Pilegaard et al., 2005) observed a prolonged and persistent (8 h) elevation in PGC1 $\alpha$  mRNA after exercise when carbohydrate availability was low. The post-

exercise glycogen content of the untrained subjects of Pilegaard et al. (2005) was similar to the values observed for the well-trained cyclists ( $\sim 350 \text{ mmol}\cdot\text{kg}^{-1} \text{ BM}$ ) in the current study. As such, it is possible that the more sustained PGC1 $\alpha$  mRNA response observed by these workers (Pilegaard et al. 2005) is due to differences in training status of the subjects, rather than the slightly longer duration of the post-exercise sampling time point the following morning (8 versus 10-12 h) in the current study.

The second bout of exercise (prolonged, submaximal steady-state cycling; 120SS) failed to elicit further increases in the phosphorylation state of AMPK<sup>Thr172</sup> or p38MAPK<sup>Thr180/Tyr182</sup> whether it was commenced after either ‘sleeping low’ or consuming an evening meal (Figure 6). However, as might be expected from the greater oxidation of fat- compared to carbohydrate-based fuels during this second exercise bout (Figure 5), ‘sleeping low’ did induce a greater increase in ACC<sup>Ser79</sup> phosphorylation. However, the responses of selected genes with roles in mitochondrial biogenesis (PGC1 $\alpha$ , Tfam or COXIV) were not substantially altered by either dietary condition in response to the second exercise bout (Figure 8). An exercise and diet-induced elevation in PGC1 $\alpha$  mRNA content was observed in the FED but not FASTED trial several hours after the completion of exercise, and at a time when carbohydrate availability was high for both conditions (from the meal consumed 60 min post-120SS). This response is difficult to explain but suggests there may be a delayed effect of withholding energy availability immediately post exercise on the adaptive responses to a subsequent training session undertaken in close proximity (i.e., within a 12 h window or even the same day). In contrast to the findings of the current study, Psilander et al., (2012) have recently reported that when muscle glycogen was depleted by prior exercise and a subsequent exercise bout commenced 14 h later with low ( $\sim 170 \text{ mmol}\cdot\text{kg}^{-1} \text{ dry wt}$ )



muscle glycogen availability, PGC1 $\alpha$  mRNA content was elevated to a greater extent than when carbohydrate was consumed during the recovery period. Differences in results between the current study and that of Psilander et al., (2012) are hard to reconcile

While 'sleep-low' failed to augment selected markers of training adaptation, lack of nutrient availability did result in marked increases in genes and/or proteins with roles in lipid utilisation. Immediately and 4 h after the second bout of exercise undertaken on day 2, PDK4 mRNA abundance was elevated to a greater extent in the FASTED compared to the FED trial. Given the significant differences in the contribution of fat and carbohydrate fuels to this exercise bout (Figure 5), this is not surprising and merely highlights the sensitivity of PDK4 to substrate availability and its role in down-regulating carbohydrate oxidation (Pilegaard and Neufer, 2004). It is clear that one of the main responses to withholding carbohydrate availability post-exercise, as well as commencing subsequent exercise with reduced muscle glycogen, is a marked elevation in fat transport and oxidation. These findings are in agreement with previous studies that have withheld post-exercise feeding (Pilegaard et al., 2005) as well as commencing exercise with reduced muscle glycogen availability (Pilegaard et al., 2002, Bartlett et al., 2013) and indeed, has been shown to result in greater fat oxidative markers of training adaptation when incorporated into a periodised training programme (Hulston et al., 2010). Need to state the findings of greater tracer-derived measured of fat oxidation by Hulston.

While the 'sleep low' protocol was specifically designed to prolong the time during which subjects were exposed to low carbohydrate availability, it was also expected that concomitant increases in systemic factors (i.e., elevated circulating FFA and catecholamine

concentrations) with putative roles in the adaptive processes (Hawley, 2011, Hawley and Burke, 2010) would be observed. However, despite a substantially greater contribution from lipid-based fuels to total energy expenditure during the prolonged steady-state ride on the morning of day 2, both circulating FFA and catecholamine concentrations were similar under both conditions (Figures 3 and 4). Hansen et al. (2005) have previously reported elevated adrenergic responses when exercise is commenced with reduced muscle glycogen. The discrepancy in findings between studies may be due to a combination of the higher starting muscle glycogen levels for the second exercise bout in the present study as well as differences in exercise mode (i.e., single-limb kicking versus whole-body exercise).

In conclusion, the results from the present study demonstrate that delaying feeding after an intense evening training session so athletes sleep with lowered carbohydrate availability results in a greater upregulation of several exercise responsive signaling markers with roles in lipid oxidation the following morning compared to when an evening meal was consumed (i.e., high overnight carbohydrate availability). Commencing prolonged, steady-state exercise after 'sleeping-low' promoted greater rates of whole-body fat oxidation, compared to 'sleeping-high' but failed to elicit a greater upregulation of cellular markers of mitochondrial biogenesis whether this novel 'train-high, sleep-low' protocol when incorporated into a periodised training program undertaken over several weeks may serve as an additional stimulus to enhance the normal adaptive responses to training remains to be determined. The results suggest that critical absolute 'thresholds' for both pre-exercise glycogen concentration and training intensity exist if specific nutrient-exercise interactions are to augment the normal training response-adaptation.

### *3.5 Acknowledgments*

The hard work and commitment of the athletes who gave their time to participate in this research project was greatly appreciated. This study would also not have been possible without the assistance of everyone from the RMIT Exercise and Nutrition Research Group who assisted in running the trials. Donny Camera is also acknowledged for his assistance in Western Blot and PCR analysis.



## ***Chapter Four***

### ***Effect of a carbohydrate mouth rinse on simulated cycling time trial performance commenced in a fed or fasted state.***

The previous two studies have investigated possible ways to enhance training adaptation under conditions of reduced CHO availability. However, during competition an athlete's priority shifts away from nutritional practices that promote adaptation and toward strategies that optimise performance. It is well known that CHO availability prior to and during competition plays a pivotal role in supporting performance. Meanwhile, it is also evident that CHO availability may influence the efficacy of potential ergogenic supplements used during competition. As such, this third study investigated the efficacy of a recently emerging ergogenic aid - a CHO mouth rinse and the effects on power output during a simulated cycling time trial commenced in a fed or fasted state.

The reference for the published version of this study is as follows:

**Lane, S. C., Bird, S. R., Burke, L. M., & Hawley, J. A. (2013).** Effect of a CHO mouth rinse on simulated cycling time-trial performance commenced in a fed or fasted state. *Appl Physiol Nutr Metab*, 38(2), 134-139.

#### *4.1 Introduction*

Performance during prolonged (>90 min) endurance sports is highly dependent upon CHO availability from both endogenous (i.e., muscle and liver glycogen) and exogenous (i.e. ingestion of CHO-containing solutions) sources (Coyle et al., 1986, Halson et al., 2004). However, even in shorter, more intense events lasting <1 h during which endogenous glycogen stores are not a limiting factor for performance (Hawley et al., 1997) there appears to be an ergogenic effect from the oral intake of CHO (Carter et al., 2004). While the precise mechanism(s) responsible for the performance enhancement associated with increased exogenous CHO availability in events <1 h are not known, it has been suggested that oral receptors within the mouth and possibly the digestive tract sense CHO and directly stimulate reward centers in the brain which increase ‘central drive’ and improve work capacity (Fares and Kayser, 2011, Chambers et al., 2009, Carter et al., 2004, Rollo et al., 2010, Pottier et al., 2010, de Salles Painelli et al., 2010, Jeukendrup and Chambers, 2010). In the pioneering study in this field, Carter et al. (2004) investigated the effects of a CHO mouth rinse (a 6.4% maltodextrin solution) or a non-CHO flavor-matched placebo rinse on 1 h cycling time trial performance in well trained cyclists. These researchers reported that the CHO-containing mouth rinse significantly improved performance compared to a placebo mouth rinse (~3%,  $P < 0.05$ ).

The findings of a ~3% performance enhancement associated with a CHO mouth rinse from this first investigation (Carter et al., 2004) is consistent with that of subsequent studies (Chambers et al., 2009, Rollo et al., 2010, Pottier et al., 2010) when subjects have commenced the performance task in a fasted state (ranging from 3-15 h). However, the effectiveness of a CHO mouth rinse to improve high-intensity performance lasting ~ 1 h when sports nutrition guidelines (Rodriguez et al., 2009) to consume a CHO rich meal 2-3 h prior to an event are followed are less clear (Beelen et al., 2009, Fares and Kayser, 2011,

Rollo et al., 2011). This is because studies to date have used a variety of different performance outcomes and exercise modes (Fares and Kayser, 2011, Chong et al., 2010, Carter et al., 2004, Rollo et al., 2010) recruited both untrained individuals and trained athletes (Fares and Kayser, 2011, Whitham and McKinney, 2007), and have fed different amounts of energy before and during the performance task (Beelen et al., 2009, Fares and Kayser, 2011, Carter et al., 2004, Pottier et al., 2010). Specifically, no study has compared the effectiveness of a CHO mouth rinse in both a fed and fasted state within the same cohort of athletes. As such, clear recommendations need to be developed that are applicable to athletes who are most likely to benefit from the use of a CHO mouth rinse. These recommendations need to accommodate the current guidelines for pre-competition nutritional practices as well as variations for athletes who may not consume a pre-event meal due to individual preferences or adverse gastrointestinal complications during exercise. Accordingly, this study sought to determine the effects of a CHO mouth rinse on a simulated high-intensity cycling time trial commenced in either a fed or fasted state. It was hypothesised that a CHO mouth rinse would enhance time trial performance in well-trained cyclists and would have a greater effect in a fasted compared to a fed state.

## 4.2 Methods

### *Subject Characteristics*

Twelve male competitive endurance trained cyclists or triathletes riding an average of  $358 \pm 61 \text{ km}\cdot\text{wk}^{-1}$  (range 285-455  $\text{km}\cdot\text{wk}^{-1}$ , values mean  $\pm$  SD) in the 6 wk prior to commencement of the study, along with a history of  $>3$  yr endurance training volunteered to participate in these trials. The subjects' age, body mass (BM), peak oxygen uptake ( $\dot{V} \text{O}_{2\text{peak}}$ ) and peak power output (PPO) were  $28 \pm 5$  yr,  $74 \pm 5.1$  kg,  $64 \pm 3.7 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  and  $397 \pm 28$  W. Prior to giving their written consent all subjects were informed of the possible risks of all procedures. The study was approved by the RMIT University Human Research Ethics Committee.

### *Preliminary Testing*

Approximately 1 wk prior to completing a familiarisation ride (described subsequently) all subjects underwent an incremental cycling test to exhaustion on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) as previously described (Hawley and Noakes, 1992). During this maximal test subjects breathed through a Hans Rudolph two-way non-rebreathing valve and mouth piece attached to a calibrated online gas system (TrueOne 2400, Parvomedics, Utah, USA) interfaced to a computer that calculated the instantaneous rates of  $\text{O}_2$  consumption ( $\dot{V} \text{O}_2$ ),  $\text{CO}_2$  production ( $\dot{V} \text{CO}_2$ ), minute ventilation ( $\text{VE}_{\text{STPD}}$ ), and the respiratory exchange ratio (RER). Before each maximal test, analysers were calibrated with commercially available gasses of known  $\text{O}_2$  and  $\text{CO}_2$  content.  $\dot{V} \text{O}_{2\text{peak}}$  was defined as the highest uptake a subject attained during any 30 s of the test while PPO was calculated from the last completed work rate plus the fraction of time



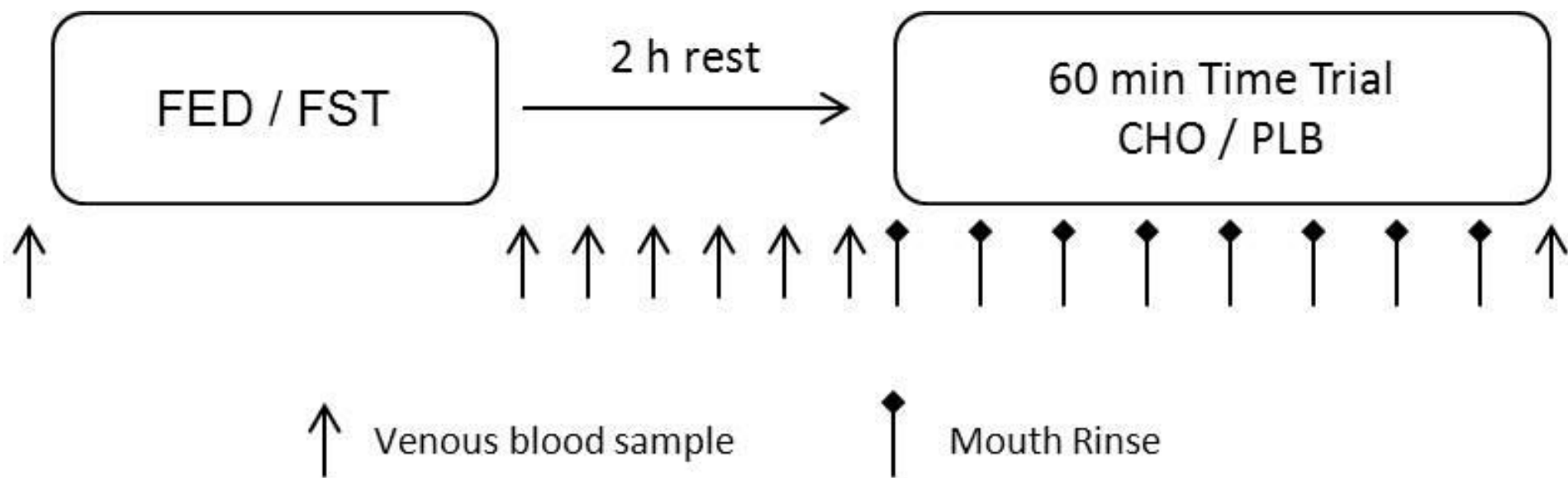
spent in the final non-completed work rate as previously described (Hawley and Noakes, 1992). The maximal test and all experimental trials were conducted under standard laboratory conditions (18-22°C, 40-50% relative humidity), and subjects were fan cooled during all exercise sessions.

### *Familiarisation to Time Trial*

One week prior to commencing their first experimental trial subjects underwent a 60 min familiarisation time trial on their own bicycles mounted on a stationary trainer (Fluid<sup>2</sup>, CycleOps, Madison, WI, USA). Each subject completed a 10 min warm up then immediately performed a 60 min time trial at their maximal self-selected power output. Power output was monitored using a PowerTap power meter (CycleOps, Saris Cycling Group, Wisconsin, USA). Subjects did not receive any feedback other than elapsed time and the percentage of time remaining. The power meter was 'zeroed' (reset) as per the manufacturer's instructions prior to each familiarisation session and subsequent experimental trials. Immediately prior to commencement of the time trial and at every 12.5% (7.5 min) of elapsed time subjects rinsed for 10 s and then 'spat' 20 mL of a solution identical to the placebo solution as later described in the experimental protocol. If the subject failed to achieve an average power output of greater than 65% of their PPO or a decline in average power of greater than 20W in the second half of the time trial they were required to return to the laboratory and undertake another familiarisation trial until the principal investigator deemed the subject was capable of reproducing consistent power outputs over the duration of the subsequent experimental trials.

### *Experimental Design*

A schematic overview of the experimental protocol is shown in Figure 4.1. In brief, subjects completed four experimental trials each separated by ~7 days. Trials were completed using a double blind (for mouth rinse), Latin Square design. Two experimental trials were commenced in a fed state (FED) and two fasted (FST). Prior to all experimental trials, subjects were provided with a standardised diet (detailed subsequently) for the day preceding the trial, which was then followed by a 9-10 h overnight fast before reporting to the laboratory between 0700-0730 h. Upon arrival, subjects rested quietly for 15 min before a teflon catheter (Terumo, 20-22G, Tokyo, Japan) was inserted into a vein in the antecubital fossa to enable repeated blood sampling (subsequently described). During the FED trials subjects then consumed a standardised high CHO breakfast containing  $2.5\text{g}\cdot\text{kg}^{-1}\cdot\text{BM}$  of CHO. This meal consisted of a mixture of glycemic index foods including cereal, milk, fruit and juice. FST trials were completed under the same conditions as FED trials but without the consumption of the standardised high CHO breakfast. The volume of water consumed during the first experimental trial was recorded and repeated for all subsequent trials.



**Figure 4.1.** Experimental Schema: FED (CHO meal;  $2.5\text{g}\cdot\text{kg}^{-1}\cdot\text{BM}$ ), FST (Overnight fast), W/U (10 min self-selected warm up), CHO (CHO mouth rinse; 8 x 20 mL of a 10% maltodextrin solution), PLB (Matched artificially sweetened solution)

### *Diet/Exercise control*

Participants were asked to abstain from all dietary sources of caffeine, alcohol and strenuous exercise for the 24 h preceding an experimental trial. Each subject's diet was 'clamped' 24 h before each experiment at an energy intake of  $0.21 \text{ MJ} \cdot \text{kg}^{-1} \text{ BM} \cdot \text{day}^{-1}$  ( $8 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  of energy from CHO;  $2.0 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  from protein and  $1.0 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  from fat). All meals and snacks were supplied to subjects with diets individualised for food preferences. Subjects received their food in pre-prepared packages and were required to keep a food checklist to note their compliance to the dietary instructions and their intake of any additional food or drinks. Subjects were allowed water *ad libitum* in the 24 h prior to and during all experimental trials.

### *Experimental trials*

Immediately after the 2 h rest period subjects performed a 10 min self-selected warm up prior to commencing a 60 min time trial. The structure of the warm up and average power output was recorded during the first trial and replicated throughout the subsequent trials. Subjects performed all trials on their own bicycles mounted on a stationary trainer as previously described. Subjects were asked to complete as much work as possible within the 60 min (i.e., maintain their highest sustainable average power output). During the time trials subjects received no feedback or motivation other than elapsed time and the total time remaining. Power output (W) was monitored using a PowerTap power meter. Mean power output (W) and heart rate (HR) were recorded at the completion of each 12.5% of total time while rating of perceived exertion (RPE) using a Borg Scale (rating of 6-20) (Scherr et al., 2013) was recorded at identical time points. To reduce any measurement variation each participant was assigned to a particular power meter and stationary trainer and completed all trials using the same equipment.

### *Mouth rinse*

To double blind the trials, an external researcher prepared the solutions and labeled each with a generic name that was only revealed after all trials were completed. Both CHO and PLB solutions were made up from a stock preparation of a commercially available non-caloric artificially sweetened and flavoured concentrate (Coola Cordial, Cottee's, Schweppes, AUS) diluted with water according to the manufacturer's directions. For the CHO trials the bolus comprised of a 10% CHO-containing maltodextrin solution that was prepared by adding maltodextrin ( $100\text{g}\cdot\text{L}^{-1}$ ) to the non-caloric stock solution. The PLB solution consisted only of the stock solution as described above. The CHO and PLB solutions were therefore matched for taste, colour and appearance. The successful blinding of these two solutions was tested prior to commencement of the experimental trials on a separate cohort of volunteers.

At the start of each time trial, each subject was given a 20 mL bolus of liquid, which they rinsed for 10 s before expectorating the bolus into a dish. Every 12.5% of total time during the time trial another 20 mL bolus of liquid was given to the subjects, with the same rinse and expectorate procedure being followed on each occasion. During the trials, subjects were also instructed not to consume any water for 5 min after each rinse so as to avoid diluting any residual CHO that may be present in the mouth.

### *Blood samples*

A total of 4 mL of whole blood was collected in tubes containing EDTA at each sampling time point (Figure 4.1), of which 25  $\mu\text{L}$  was immediately analysed for glucose concentration (YSI, Yellow Springs, Ohio, USA). For all trials a resting blood sample was taken upon

presenting to the laboratory. In the FED trials subsequent sampling began 20 mins after the commencement of the pre-trial meal and was repeated every 20 min thereafter during the 2 h rest prior to the time trial. During the FST trials samples were taken at the equivalent time point. Immediately after the completion of each time trial a final sample was collected. After each sample collection the catheter was flushed with 2-3 mL of 0.9% sterile saline to ensure patency of the vein.

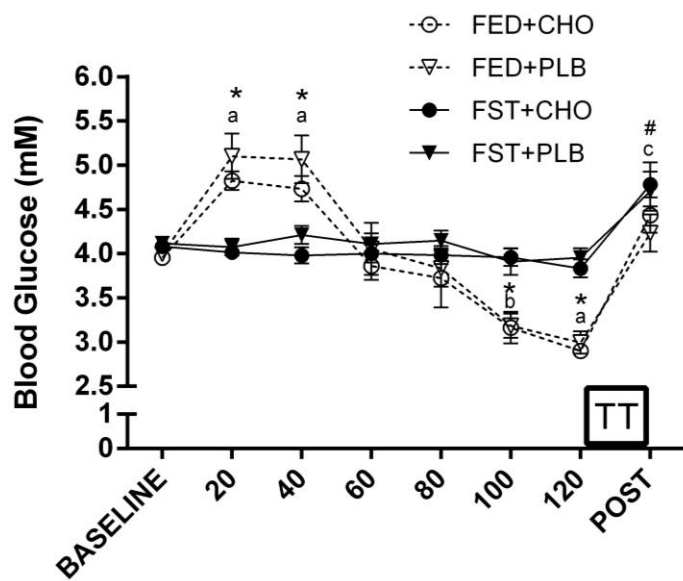
### *Statistical Analysis*

Statistical analyses were performed using software package SPSS (Version 18). Main effects of the rinse and prandial state were analysed using two-way repeated measures ANOVA's. One-way ANOVA's for repeated measures were used to compare between time points and trials using a Bonferroni adjustment. Significance was set at  $P < 0.05$ . All data are presented as mean  $\pm$  SEM unless otherwise stated.

### 4.3 Results

#### *Blood glucose concentration*

Blood glucose concentrations for the four experimental trials are shown in Figure 4.2. Blood glucose levels recorded upon arrival at the laboratory were similar for all trials. In both the FST trials blood glucose concentrations remained at ~4mM throughout the 2 h rest that preceded the time trials. Conversely in both FED trials, blood glucose significantly increased after breakfast and remained elevated for 40 min. After this time, blood glucose concentrations in the FED trials declined and returned to near resting levels at 60 min. They then continued to drop and were below resting levels at the commencement of the time trials. Statistical analyses confirmed that at the beginning of the warm up, blood glucose concentrations were significantly different between FED and FST trials ( $P < 0.01$ ). At the end of the time trial all trials displayed a blood glucose concentration greater than that recorded pre warm-up ( $P < 0.05$ ). Additionally, at the end of the time trial the blood glucose levels for FED+PLB trial were statistically ( $P < 0.05$ ) lower than those for either of the FST trials. However, the magnitude of this difference was small, and at this time point all trial conditions displayed similar blood glucose levels (4.2 – 4.8 mM).

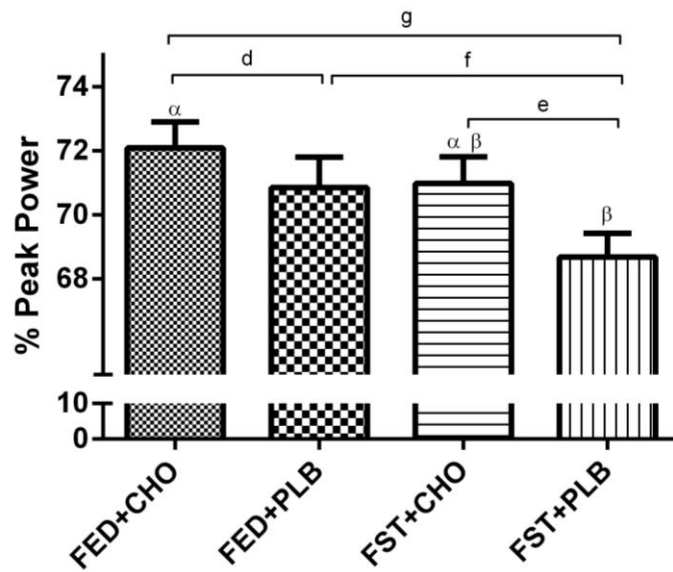


**Figure 4.2.** Blood glucose concentrations: FED+CHO (Fed with carbohydrate mouth rinse); FED+PLB (Fed with placebo mouth rinse); FST+CHO (Fasted with carbohydrate mouth rinse); FST+PLB (Fasted with placebo mouth rinse); (\*) FED trials different to FST trials; (#) FED+PLB different to FST trials, (a) different to BASELINE in FED trials, (b) different to BASELINE in FED+PLB, (c) different to pre warm-up (120) for all trials; Mean  $\pm$  SEM,  $P < 0.05$ .

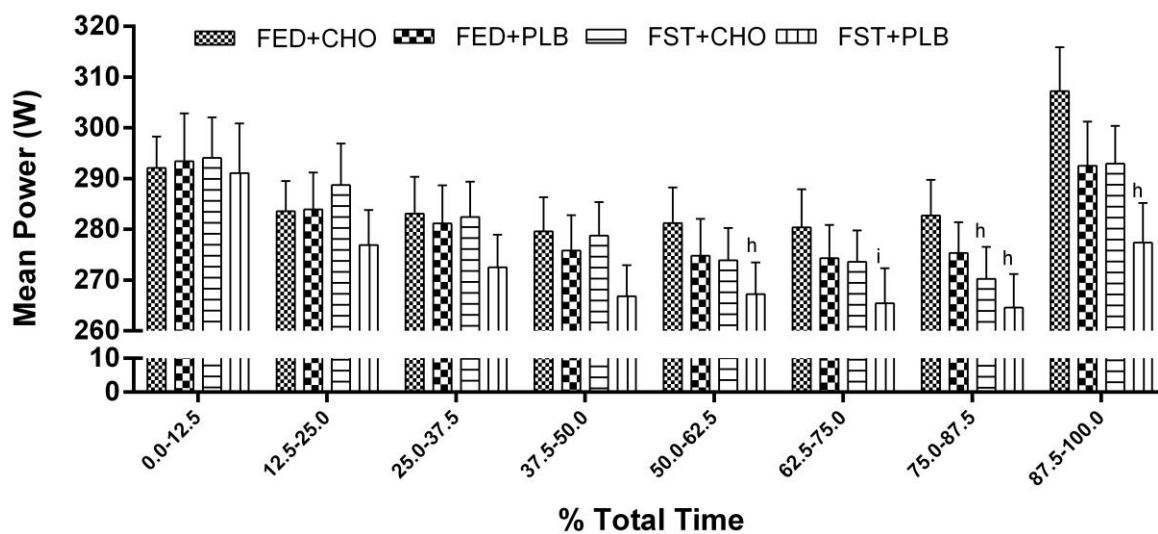


### *Power Output*

There was a significant main effect of the CHO mouth rinse ( $P = 0.001$ ) and prandial state ( $P = 0.002$ ) on mean power output (Figure 4.3). There was an interaction between the two variables ( $P = 0.015$ ) suggesting that pre-feeding influences the magnitude of effect of the mouth rinse procedure. In summary, the CHO mouth rinse improved mean power output by 1.8% ( $286 \pm 6$  W vs  $281 \pm 5$  W;  $P < 0.05$ ) and 3.4% ( $282 \pm 6$  W vs  $273 \pm 6$  W;  $P < 0.01$ ) for the FED and FST trials respectively. Whereas commencing the time trial in a FST state reduced mean power output by 1.6% ( $P = 0.14$ ) and 3.2% ( $P < 0.01$ ) for the CHO and PLB trials respectively. Further analysis, comparing the mean power output for each 12.5% segment (Figure 4.4), revealed that there were no differences between trials at any segment up until 50-62.5% of total time completed. After this, the mean power during all segments was lower in the FST+PLB trial compared to the FED+CHO trial. At 62.5-75% of total duration mean power for FST+PLB was significantly lower compared to both FED+CHO and FED+PLB, and during the 75-87.5% segment FST+CHO was also lower than FED+CHO. The significant interaction (prandial state (FED v FST) x mouth rinse (CHO v PLB)) indicated that the CHO mouth rinse produced greater improvements in mean power output in the FST state compared to the FED prandial state.



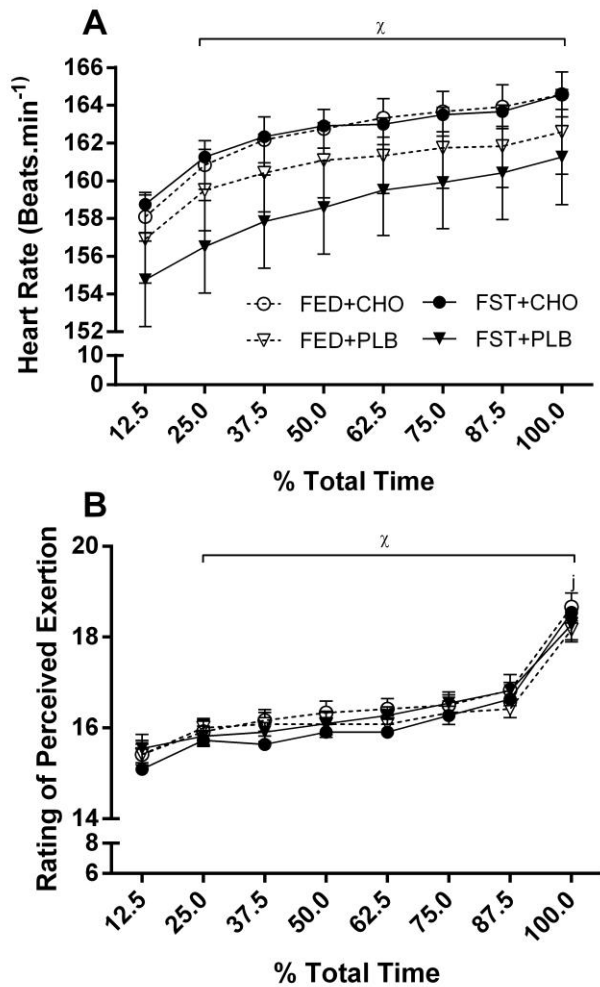
**Figure 4.3.** Mean power output: FED+CHO (Fed with carbohydrate mouth rinse); FED+PLB (Fed with placebo mouth rinse); FST+CHO (Fasted with carbohydrate mouth rinse); FST+PLB (Fasted with placebo mouth rinse); ( $\alpha$ ) Main effect of CHO rinse ( $P < 0.001$ ); ( $\beta$ ) Main effect of FST ( $P = 0.003$ ); (d) FED+CHO different to FED+PLB ( $P = 0.024$ ), (e) FST+CHO different to FST+PLB ( $P = 0.001$ ), (f) FED+PLB different to FST+PLB ( $P = 0.005$ ), (g) FED+CHO different to FST+PLB ( $P > 0.001$ ); Mean  $\pm$  SEM,  $P < 0.05$ .



**Figure 4.4.** Mean power output for each 12.5% of total duration. FED+CHO (Fed with carbohydrate mouth rinse); FED+PLB (Fed with placebo mouth rinse); FST+CHO (Fasted with carbohydrate mouth rinse); FST+PLB (Fasted with placebo mouth rinse); (h) different to FED+CHO; (i) different to FED+CHO and FED+PLB; Mean  $\pm$  SEM,  $P < 0.05$ .

### *Heart rate and rating of perceived exertion*

There was a main effect for time on HR ( $P = 0.02$ ) and RPE ( $P = 0.002$ ) with values steadily increasing throughout the time trials (Figure 4.5). There were no differences between trials at any time point for both HR and RPE. During the final 12.5% (87.5-100%) of total time RPE was significantly higher in all trials when compared to all other time points.



**Figure 4.5.** Characteristics during the 1 h time trial: FED+CHO (Fed with carbohydrate mouth rinse); FED+PLB (Fed with placebo mouth rinse); FST+CHO (Fasted with carbohydrate mouth rinse); FST+PLB (Fasted with placebo mouth rinse); A) Accumulated mean heart rate, B) Rating of Perceived Exertion. ( $\chi$ ) Main effect of time ( $P < 0.05$ ), (j) different to all other time points for all trials ( $P < 0.01$ ); Mean  $\pm$  SEM,  $P < 0.05$ ).

### *Rinse solution detection*

After the completion of each time trial, subjects were asked to identify which solution they believed they had received that day. Out of a total of 48 time trials completed, subjects selected the correct solution on 21 occasions (i.e. less than would be predicted by pure chance). Three subjects correctly identified the solutions administered in all 4 trials, although only two of these subjects performed better in both CHO trials when compared to the respective PLB trials.

#### *4.4 Discussion*

This study determined the effect of a CHO mouth rinse on 1 h simulated cycling time trial performance commenced in a fed or fasted state in competitive male cyclists/triathletes. It was hypothesised that a CHO-containing rinse would improve mean power output in the cohort of well-trained athletes, but, in accordance with the results of previous studies (Beelen et al., 2009, Carter et al., 2004, Fares and Kayser, 2011, Pottier et al., 2010) this effect would be greater when the time trial was commenced in a fasted state. The results revealed several findings on which practical recommendations can be based: 1) Consuming a CHO-rich meal ( $2.5 \text{ g}\cdot\text{kg}^{-1} \text{ BM}$ ) two hours prior to a time trial enhanced mean power output compared to undertaking the time trial in a fasted state; 2) regular use of a CHO mouth rinse throughout a time trial increases mean power output compared with mouth rinsing with a placebo, by a magnitude equal to the effect of consuming the pre-event meal; 3) the CHO mouth rinse improved power output during the time trial both after an overnight fast and when the time trial was commenced after a high CHO meal. However, enhancement of performance was greater when undertaken in the fasted state (3.4%) than compared to the fed state (1.8%) and; 4) the best performance outcome was achieved by the combination of both a CHO-rich pre-event meal and the regular use of the CHO mouth rinse throughout the time trial.

The finding of a ~3% improvement in power output in a fasted state is consistent with that of other studies that have employed well-trained subjects and cycling time trials lasting ~1 h (Carter et al., 2004, Pottier et al., 2010, Chambers et al., 2009). However, they are in contrast to those of Beelen et al., (2009) who used a similar pre-feeding intervention (~ $2.5 \text{ g}\cdot\text{kg}^{-1} \text{ BM}$  consumed 2 h prior to the cycling time trial) yet reported no benefit of a CHO mouth rinse. Differences in results between the two investigations are hard to reconcile given that both studies utilised well-trained cyclists and had similar experimental protocols. The only variation appears to be the composition, concentration and duration of the mouth rinse, with

Beelen et al. (2009) using a 6.4% maltodextrin solution and water placebo rinsed for 5 sec compared to 10% maltodextrin solution rinsed for 10 sec in the present study. Such differences suggest that the concentration and duration of oral contact within the mouth rinse may have an impact on its effectiveness. The present study used a CHO solution of greater concentration (Beelen et al., 2009, Carter et al., 2004, Chong et al., 2010, Fares and Kayser, 2011, Pottier et al., 2010, Rollo et al., 2010, Rollo et al., 2008, Rollo et al., 2011, Whitham and McKinney, 2007) and rinse duration (Beelen et al., 2009, Whitham and McKinney, 2007, Rollo et al., 2011, Rollo et al., 2010, Pottier et al., 2010, Chong et al., 2010, Carter et al., 2004) compared to previous studies. Subjects were also specifically instructed not to consume water for a 5 min period after each rinse to prolong the possible activation of oral receptors resulting from any residual CHO remaining in the mouth although, further work would be needed to directly test this hypothesis.

Fares and Kayser (2011) studied the interaction of pre-feeding and a similar mouth rinse to that used in the current study in a non-athletic cohort. Specifically, they investigated the effects of a CHO mouth rinse on cycling time to fatigue (at 60%  $W_{max}$ ) in fed and fasted condition and found that time to exhaustion was increased by 3% and 7% in the fed and fasted states compared with the corresponding placebo trial. The current results support and extend these findings by clearly demonstrating that a CHO mouth rinse can improve performance in well-trained cyclists under situations that more closely simulate competitive cycling (time trial vs time to fatigue) even when a CHO meal is consumed within 2-3 h of an event. Perhaps the most practical approach to enhance performance capacity would be to CHO mouth rinse then ingest (as opposed to expectorate) a small (20-25 mL) volume of a CHO solution. Certainly this strategy could be applied over a range of sporting situations and may avoid the complications associated with gastrointestinal distress common when larger volumes of fluid are ingested.



The mechanism of the diminished effectiveness of a CHO mouth rinse on performance in a FED state is yet to be clearly identified. However, there is some evidence that the degree of activation of reward centres in the brain described by Chambers et al. (2009) is influenced by background feelings of hunger and satiety. For example, Haase and colleagues (2009) reported significant differences in activation of the insula under conditions of hunger and satiety when a sucrose solution ( $219 \text{ g}\cdot\text{L}^{-1}$ ; 22% CHO) was administered to the tongue under resting conditions. Their observation of a greater activation of the primary taste cortex in a fasted state is consistent with the current findings of greater effectiveness of a CHO mouth rinse on cycling performance undertaken under these same conditions and merits further investigation. It would be of interest, for example, to determine the latency period or period of deprivation of CHO intake that is required before differences in cortical responses to CHO sensing can be detected.

The CHO-rich meal ( $2.5 \text{ g}\cdot\text{kg}^{-1} \text{ BM}$ ) in the FED trials resulted in a rapid rise in blood glucose concentrations, with peak levels occurring between 20 and 40 min after ingestion. The observed decline in blood glucose concentrations over the remaining rest period (~80 min) to levels approaching hypoglycemia (~3.5 mM) is a consistent observation (Brouns et al., 1989, Costill et al., 1973, Seifert et al., 1994, Short et al., 1997, Foster et al., 1979, Moseley et al., 2003). This response has been coined 'relative transient hypoglycemia' (Costill et al., 1973) and it appears to be rescued upon commencement of exercise (Moseley et al., 2003). Although similar blood glucose concentrations at the completion of the time trial between treatments was observed, it can only be speculated that the blood glucose concentrations in FED trials were normalised to that of FST trials upon commencement of the time trial, as a result of the pre-time trial warm up. Nevertheless, it should be remembered that even if hypoglycemia did occur transiently in the FED trials, performance was better under these conditions than the FST trials. The current findings also revealed that despite the possible

differences in blood glucose concentrations between FED and FST at the commencement of the performance bout, RPE and power output were very similar between all trials in the first quarter of the time trial. These findings are in agreement with Moseley et al. (2003) who reported that despite differences between trials in blood glucose concentrations at the commencement of a ~40 min time trial (as a result of different timings of pre-event feeding) there were no differences in RPE, HR,  $\dot{V}O_2$  or performance.

Analysis of mean power output for each 12.5% of total time revealed no marked differences between trials until the completion of almost two-thirds of the ride (62.5%, 37.5 min). In the latter third of the time trial there was a trend for mean power to be higher in FED compared to its corresponding FST trial and for the mouth rinse to become more effective with time throughout the ride. It is well established that CHO availability is closely linked to self-selected maximal intensities (Hulston et al., 2010, Yeo et al., 2010, Yeo et al., 2008b, Rollo et al., 2011) and ratings of perceived exertion during prolonged, constant-load, submaximal exercise (Coyle et al., 1986). It can be speculated that the reduction in motor output as a protective mechanism resulting from central feedback of CHO availability (liver, muscle and blood) can be ameliorated by the central stimulatory effect of a CHO mouth rinse. In addition, it would appear that the greater the reduction in motor output as a result of feedback mechanisms monitoring CHO availability the greater the effectiveness of a CHO mouth rise. Interestingly, this modulation of central output and or feedback improved power output without an increase in perceived effort. Such an observation is supported by others (Rollo et al., 2010, Carter et al., 2004, Chambers et al., 2009) and further studies will need to be undertaken to identify the precise mechanism(s) responsible for the dissociation between exercise intensity and perceived exertion when a CHO mouth rinse is used.

One limitation of this study was the inability to avoid any possible ‘nocebo’ effect of the fasted state. Because each individual was aware of the FED or FST manipulation it cannot be overlooked that potential prior beliefs that the fasted state may be detrimental to performance may have affected the benefits of the CHO mouth rinse procedure.

In conclusion this study has shown that regular mouth rinsing with a CHO-containing solution during intense exercise lasting ~1 h enhances power output in both a fed and fasted state. However, the effectiveness of a CHO mouth rinse is greater when cycling is commenced in a fasted compared to the fed state. The findings suggest that optimal performance is achieved with the combination of a high CHO pre-event meal and the CHO mouth rinse procedure. This study also provides practical recommendations for athletes who suffer gastrointestinal problems as a result of pre- or during-event feeding strategies or where training constraints (ie, training early in the morning prior to breakfast) may still benefit from the use of a CHO mouth rinse at regular intervals throughout training and competition.

#### *4.5 Acknowledgments*

The hard work and commitment of the athletes who gave their time to participate in this research project was greatly appreciated. This study would also not have been possible without the assistance of everyone from the RMIT Exercise and Nutrition Research Group who assisted in running the trials.

## ***Chapter Five***

### ***Single and combined effects of beetroot juice and caffeine supplementation on cycling time trial performance.***

The final study presented as part of this thesis aimed to further investigate the efficacy of known ergogenic supplements under nutritional conditions known to support optimal performance. Findings from previous studies of this thesis that were shown to be optimal to performance, including the use of caffeine as well as the ingestion of a pre-event meal in combination with regular oral CHO contact were incorporated into this study. With this background of nutritional/ergogenic strategies that are typically used by elite endurance athletes, this study's main aim was to determine if beetroot juice, a contemporary ergogenic supplement when combined with caffeine would provide an additive ergogenic effect during a performance trial replicating the demands of the 2012 London Olympic cycling time trial.

The reference for the published version of this study is as follows:

**Stephen C. Lane**, John A. Hawley, Ben Desbrow, Andrew M. Jones, James R. Blackwell, Megan L. Ross, Adam J. Zemski, Louise M. Burke. Single and combined effects of beetroot juice and caffeine supplementation on cycling time trial performance. *Appl Physiol Nutr Metab*. Accepted Oct 11 2013.

### *5.1 Introduction*

Athletes are continually striving to improve training capacity and performance. Not surprisingly, widespread use of a large number of nutritional supplements is commonplace in most sports as athletes search for a ways that will elevate their performance to a higher level. Both caffeine (Lane et al., 2013a, Irwin et al., 2011, Desbrow et al., 2009) and nitrate ( $\text{NO}_3^-$ ) (Cermak et al., 2012a, Vanhatalo et al., 2011, Lansley et al., 2011a) have been shown to improve simulated road cycling performance in a variety of protocols. Through mechanisms likely related to the central nervous system (CNS) (Costill et al., 1978, Tarnopolsky 2008) caffeine has been shown to improve arousal states (Backhouse et al., 2011) and reduce perceived exertion during steady state exercise (Backhouse et al., 2011, Doherty and Smith, 2005, Lane et al., 2013a) resulting in enhanced performance during sustained high-intensity cycling events (Cox et al., 2002, Lane et al., 2013a, McNaughton et al., 2008). Accordingly, contemporary protocols for caffeine use are based on evidence that moderate intakes ( $3 \text{ mg}\cdot\text{kg}^{-1}$ ) of caffeine are equally as effective as larger doses ( $6 \text{ mg}\cdot\text{kg}^{-1}$ ) (Desbrow et al., 2012) for eliciting these CNS effects, and that caffeinated gums can also provide a rapidly absorbed caffeine dose (Ryan et al., 2013, Kamimori et al., 2002). With regard to dietary  $\text{NO}_3^-$  supplementation, Jones and co-workers (Bailey et al., 2010, Bailey et al., 2009, Vanhatalo et al., 2011, Lansley et al., 2011b, Lansley et al., 2011a) have reported that ingestion of beetroot juice increases exercise capacity through metabolic mechanisms that improve contraction efficiency within skeletal muscle. It was hypothesised that the increased CNS drive and reduced perceived exertion elicited by caffeine supplementation in combination with the previously reported improvements in metabolic efficiency resulting from beetroot juice ingestion would result in higher sustainable power outputs than when each supplement was taken in isolation.

The specific aim of this study was to investigate the independent and combined effects of caffeine and  $\text{NO}_3^-$  supplementation on the performance of a cycling task simulating the physical challenges of the London 2012 Olympic Games Road Cycling Time trial (TT). These effects were investigated against the background of a standardised dietary preparation including strategies that are typical of TT specialists; these included the intake of a small volume of fluid during the event and frequent mouth contact with CHO in the form of a sports confectionary, a practice recently confirmed as being beneficial to performance (Carter et al., 2004, Chambers et al., 2009, Lane et al., 2013b, Pottier et al., 2010), even when preceded by a CHO-rich pre-event meal (Lane et al., 2013b). Additionally we aimed to investigate any potential differences in effects between male and female athletes. It was hypothesised that under optimal nutritional conditions i) caffeine alone and ii)  $\text{NO}_3^-$  alone supplementation would improve TT performance and iii) the concurrent use of caffeine and  $\text{NO}_3^-$  supplementation would result in an additive performance enhancement that was greater than when each supplement was used in isolation.

## **5.2 Methods**

### *Subjects*

Twelve male [mean  $\pm$  SD; age  $31 \pm 7$ , body mass (BM)  $73.4 \pm 6.8$  kg, height  $180.8 \pm 6.1$  cm, maximal aerobic power (MAP)  $459.4 \pm 31.1$  W, peak oxygen consumption ( $\dot{V} \text{O}_{2\text{peak}}$ )  $71.6 \pm 4.6$  mL $\cdot$ kg $^{-1}\cdot$ min $^{-1}$ ] and 12 female [age  $28 \pm 6$ , BM  $62.1 \pm 8.9$  kg, height  $169.1 \pm 8.0$  cm, MAP  $327.1 \pm 32.3$  W,  $\dot{V} \text{O}_{2\text{peak}}$   $59.9 \pm 5.1$  mL $\cdot$ kg $^{-1}\cdot$ min $^{-1}$ ] competitive cyclists or triathletes volunteered to participate in this study. Ethical clearance was obtained from the Australian Institute of Sport Ethics committee. Prior to participation, subjects were informed of the

nature and risks involved and completed a medical questionnaire before providing written informed consent.

### *Study overview*

On separate days following familiarisation (described subsequently), subjects performed four cycling time trials under different experimental conditions: Caffeine and beetroot juice supplementation (CAFF+BJ), caffeine and placebo beetroot juice (CAFF), beetroot juice and placebo caffeine (BJ) or a control trial consisting of a placebo of both caffeine and beetroot juice (CONT). All trials were separated by ~7 days, and treatments were allocated using a double-blind Latin square design. Each ride was performed under standardised conditions representing optimal nutritional practice: CHO-rich 'pre-event meal', ingestion of small amounts of a CHO-electrolyte drink during the TT, and regular oral CHO contact in the form of a sports confectionery product. All preliminary testing and experimental trials were performed under standard laboratory environmental conditions (STPD).

### *Incremental cycle test*

In the 2 wk prior to their first experimental trial all subjects performed a progressive maximal exercise test to exhaustion on a cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). After a 5 min warm up, the test protocol commenced at 175 and 125 W for males and females respectively and increased by 25 W every 60 s until volitional fatigue. Maximal aerobic power (MAP) was determined as the power output of the highest stage completed plus the fraction of any uncompleted workload as previously described (Ross et al., 2012, Ross et al., 2011). Expired gases were collected into a calibrated and customised Douglas bag gas analysis system, which incorporated an automated piston that allowed the



concentrations of O<sub>2</sub> and CO<sub>2</sub> (AEI Technologies, Pittsburg, PA) and volume of air displaced, to be quantified. The operation and calibration of this equipment have been described previously (Russell et al., 2002). Peak oxygen uptake ( $\dot{V} O_{2peak}$ ) was calculated as the highest average O<sub>2</sub> consumption recorded over 60 s.

### *Familiarisation session*

On the same day as the maximal test, subjects completed a familiarisation ride on the same bike and simulated course they would complete in the subsequent experimental trials. In brief, subjects completed the course at their own self-selected intensity with the instruction to familiarise themselves with the course profile, bike set-up and the maximal intensity they believed they could sustain for the entire duration of the TT during subsequent rides. During this familiarisation, dimensions for the bike set-up were recorded for replication throughout all experimental trials. Subjects were also familiarised with the use of the sports confectionery product (described subsequently) to be used during the experimental trials.

### *Diet/Exercise control*

Subjects consumed a standardised diet for the 24 h period prior to each experimental trial using a pre-packaged standardised diet protocol described previously (Jeacocke and Burke, 2010). Dietary goals for this period were 8 g·kg<sup>-1</sup> BM CHO; 1.5 g·kg<sup>-1</sup> BM protein; 1.5 g·kg<sup>-1</sup> BM fat; and ~220 kJ·kg<sup>-1</sup> BM for the 24 h period. Subjects were instructed to avoid alcohol for the 24 h prior, and follow their habitual caffeine consumption patterns until 12 h prior to the start of the TT. Caffeine was not withheld for the 24 h period, since it has previously been shown a 3 mg·kg<sup>-1</sup> BM dose of caffeine improves cycling performance irrespective of

whether a withdrawal period is imposed on habitual caffeine users (Irwin et al., 2011). The provided pre-trial standardised diets did not contain any  $\text{NO}_3^-$  rich products to avoid any possible effect on the experimental trials.

A food menu was prepared for each subject based on individual BM and food preferences following an initial interview with a sports dietitian (AZ). During the same consultation, subjects reported the ongoing or acute use of any known medicine or supplement. In any case where the subject reported the use of a known medicine or supplement that may influence performance between trials, the subject was excluded from the study. The subjects' individual menu was prepared using *Food Works Professional Edition, Version 6.0.2562* (Xyris Software, Brisbane, Australia). Subjects were provided with all foods and drinks in portion controlled packages for consumption during the first 22 h of the dietary control period, and were given verbal and written instructions on how to follow the diet. Checklists were used to record each menu item as it was consumed and to note any deviations from the menu. Each subject's food checklists were checked and clarified for compliance to the standardisation protocols by the sports dietitian prior to undertaking each trial. Analysis of the actual diet consumed by the subjects was undertaken on completion of the study using the same software.

### *Experimental trials*

Subjects presented to the laboratory on four separate occasions each separated by ~7 d. On each occasion, subjects presented at the same time of day, voided their bladder prior to having their BM recorded then rested in a supine position for ~10 min. At this time a Teflon cannula (Terumo, 20-22G, Tokyo, Japan) was inserted into a vein in the antecubital fossa. A resting blood sample (8 mL) was taken and the cannula flushed with saline to keep the vein

patent for subsequent sampling. Two hours prior to the warm-up for each trial and immediately after the resting blood sample subjects consumed the remainder of the control diet as a 'pre-race' meal. This meal provided 2 g·kg<sup>-1</sup> BM CHO which was included in the total CHO quota in the 24 h standardised diet. Subjects were instructed to consume their 'pre-race' meal within 20 min, after which time they remained in the laboratory for the duration of that day's experimental trial. Depending upon the trial, either the experimental or placebo beetroot juice concentrate was ingested in two separate doses (detailed below). Forty minutes prior to commencement of the TT subjects completed a standardised warm-up on the same bicycle as they performed the TT. The caffeine gum was administered in two doses, the first immediately prior to commencement of the warm up and the second immediately after its completion. Subjects then completed a TT simulating the characteristics of the London Olympic Games cycling TT course specific to the male or female events under the conditions as described below. Mean power output, heart rate and rating of perceived exertion were recorded during each trial. During the first trial water was provided *ad libitum* for the time period leading up to commencement of the TT. The volume consumed in this period was recorded and replicated throughout subsequent trials.

### *Warm-up*

The warm-up consisted of 30 min cycling at varying intensities (13 min at 25%, 5 min at 60%, 2 min at 70%, 3 min at 25%, 5 min at 60% and 2 min at 80% of MAP). Subjects then rested for 10 min prior to commencing the TT.

### *Time Trials*

Subjects performed all experimental trials on a Velotron cycle ergometer (Racermate, Seattle, WA, USA) adjusted to the dimensions of their own bicycles. Males completed a simulated 43.83 km course while females completed a 29.35 km course. The courses were created using global positioning satellite (GPS) data collected during a prior reconnaissance of the London Olympic TT race. Subjects were instructed to complete the TT as quickly as possible. Financial incentives were offered to encourage maximal effort.

### *Experimental Interventions*

#### *Beetroot Juice*

During two of the trials subject's received two separate doses of 140 mL of concentrated  $\text{NO}_3^-$  rich beetroot juice delivering 8.4 mM of  $\text{NO}_3^-$  in each dose (*Beet it*, James White Drinks Ltd., Ipswich, U.K.). Each subject ingested the first dose at a specific time ~8-12 h prior to the commencement of each TT and this was provided within each subjects controlled diet that was consumed the day prior to each experimental trial. The second dose was ingested in the laboratory 130 min prior to the commencement of the TT. During the two placebo trials, a similar tasting but  $\text{NO}_3^-$  depleted beetroot juice product (~0.006 mM of  $\text{NO}_3^-$ ; *Beet it*, James White Drinks Ltd., Ipswich) was administered at identical time points as for the experimental trials.

### *Caffeine*

During the two caffeine trials a caffeinated gum (*Stay Alert*, Amurol Confectioners, Yorkville, IL, U.S.A.) was administered in 2 doses to deliver a total of  $3 \text{ mg} \cdot \text{kg}^{-1}$  BM of caffeine. The gum was administered in a non-transparent package emptied directly into the mouth to avoid possible visual cues about the differences between trials (experimental vs. placebo). The first dose was administered immediately prior to the commencement of the warm up (40 min prior to the TT) and consisted of a caffeine dose containing  $2 \text{ mg} \cdot \text{kg}^{-1}$  BM. Subjects were instructed to chew the gum for a total of 10 min before it was removed and discarded. The remaining dose containing  $1 \text{ mg}^{-1} \cdot \text{kg}^{-1}$  BM was administered under the same instructions at the end of the warm up (10 min prior to the TT). During the placebo trials, non-caffeinated gum matched for taste and texture (*Jila Gum*, Ferndale Confectionary Pty Ltd, Australia) was provided under the same conditions as the caffeinated gum.

### *CHO Ingestion*

To ensure the findings of this study were relevant when applied in a ‘real world’ situation in which athletes follow current nutritional guidelines to maximise performance, a carbohydrate sports gel (PowerBar Gel; Powerbar Inc, Florham Park, NJ) containing 28 g of CHO was ingested 15 min prior to the commencement of each TT. Additionally, at the commencement of each TT subjects were provided with a sports confectionary product (PowerBar Gel Blasts; Powerbar Inc, Florham Park, NJ, U.S.A.). Subjects were instructed to place the confectionery item in their mouth and leave it in their cheek cavity until it had completely dissolved, at which time another was provided. The timing and number of confectionery pieces used in the

first trial was replicated throughout all subsequent trials. The aim of this procedure was to provide a constant CHO stimulus in the mouth similar to a CHO mouth rinse that has previously been shown to enhance cycling performance (Carter et al., 2004, Chambers et al., 2009, Fares and Kayser, 2011, Pottier et al., 2010, Lane et al., 2013b). Subjects also received a CHO-electrolyte ‘sports drink’ (Gatorade; Gatorade Co, Chicago, IL, U.S.A.) to consume at specific points during each TT. During the first trial males received two bottles, the first at 15 km and the second at 30 km during the TT whereas females received a single bottle at 15 km. These points correspond to portions of the TT in which prior reconnaissance of the course suggested it would be practical for competitors to take a drink. During the first trial, each bottle was pre-weighed and subjects instructed to consume as much fluid as desired within 1 min. Each bottle was then re-weighed and the volume of fluid consumed was recorded and repeated throughout all subsequent trials.

#### *Blood collection and analysis*

At each sampling time point a total of 8 mL of whole blood was collected in a tube containing lithium heparin. Each trial included four sampling time points consisting of a resting sample, one immediately prior to commencement of the warm up (prior to caffeine ingestion), a third immediately post the warm up and a final sample taken immediately post the TT. Tubes were immediately centrifuged at 4°C at 4000 rev·min<sup>-1</sup> (3040 x g) for 10 min. The resultant plasma was divided into equal aliquots and stored at -80 °C for the subsequent analysis of caffeine, NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> concentrations.

#### *Plasma caffeine concentration*

The quantitative analysis of plasma caffeine was performed using an automated ‘reverse phase’ high-performance liquid chromatography system. Conditions were adapted with subtle modifications from Koch, Tusscher, Koppe and Guchelaar (Koch et al., 1999). The precise method has been described by us previously (Desbrow et al., 2009).

#### *Plasma NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> concentration*

Plasma NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> were analysed by gas phase chemiluminescence analysis. This initially required NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> to be reduced to nitric oxide (NO) gas. For reduction of NO<sub>2</sub><sup>-</sup>, undiluted plasma was injected into a glass purge vessel containing 5 mL glacial acetic acid and 1 mL NaI solution. For NO<sub>3</sub><sup>-</sup> reduction, plasma samples were deproteinised in an aqueous solution of zinc sulphate (10% w/v) and 1M sodium hydroxide, prior to reduction to NO in a solution of vanadium (III) chloride in 1 M hydrochloric acid (0.8% w/v). Quantification of NO was enabled by the detection of light emitted during the production of nitrogen dioxide formed upon reaction of NO with ozone. Luminescence was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence nitric oxide analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). The concentrations of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> were determined by plotting signal area (mV) against a calibration plot of 25 nM to 1 µM sodium nitrite and 100 nM to 10 µM sodium NO<sub>3</sub><sup>-</sup> respectively.

#### *Statistical Analysis*

Statistical analyses were performed using software package SPSS (Version 18). For all blood and physiological measures (combining both males and female results) one-way ANOVA’s

for repeated measures were used to compare between time points and trials using a Bonferroni adjustment where appropriate. Mean power output from the four trials were analysed for males and females separately as well as combined using the magnitude based inference approach recommended for studies in sports medicine and exercise (Hopkins et al., 2009). The same inference based approach was also used to compare time to complete each trial for males and females separately. A spread sheet (Microsoft Excel), designed to examine post-only crossover trials, was used to determine the clinical significance of each treatment (available at [newstats.org/PostOnlyCrossover.xls](http://newstats.org/PostOnlyCrossover.xls)), as based on guidelines outlined by Hopkins (Hopkins, 2007). Qualitative inferences are reported as the percentage chance of a positive effect compared to the corresponding trial where a least worthwhile effect on power output of 1% was used as previously established (Paton and Hopkins, 2006). Significance was set at  $P < 0.05$ . All data are presented as mean  $\pm$ SD unless otherwise stated.

### **5.3 Results**

#### *Body mass*

There was no difference in BM upon presenting to the laboratory between trials. Similarly there was no statistical difference between trials for the change in BM pre- and post- each trial (Table 5.1).

#### *Plasma caffeine*

Figure 5.1A displays the plasma caffeine concentrations for all trials. At rest there was a small variation in plasma caffeine concentrations, likely because subjects were only instructed to abstain from caffeine in the 12 h prior to trials (Irwin et al., 2011). Within 30

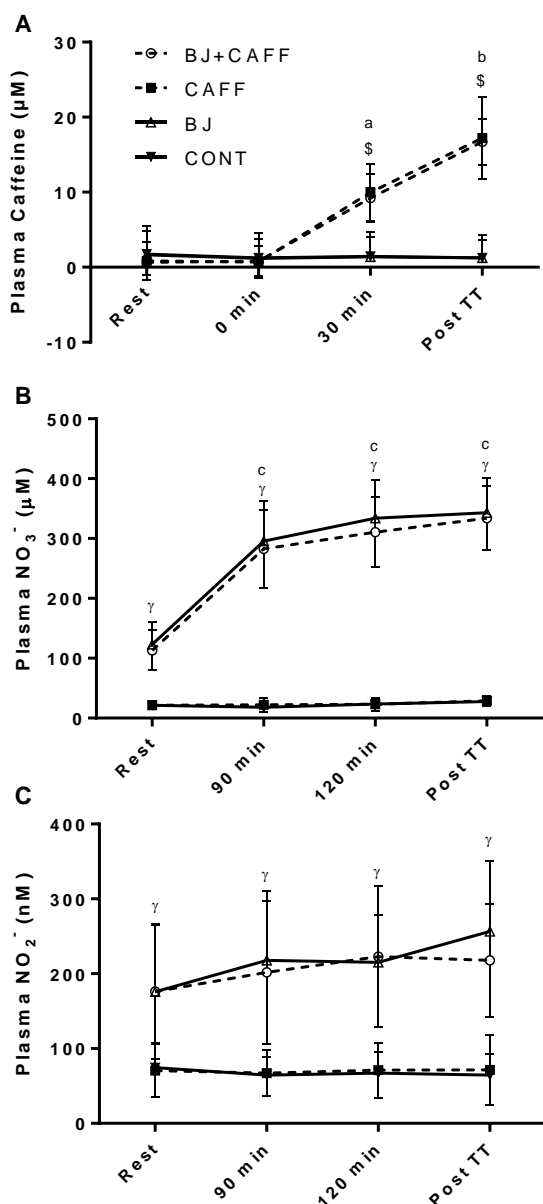


min of ingestion plasma caffeine concentrations were significantly increased (CAFF+BJ  $9.2 \pm 3.2$ , CAFF  $10.0 \pm 3.80 \mu\text{M}\cdot\text{L}^{-1}$ ) compared to resting values and when compared to the non-caffeine trials. Peak caffeine concentrations (CAFF+BJ  $16.7 \pm 3.1$ , CAFF  $17.2 \pm 5.5 \mu\text{M}\cdot\text{L}^{-1}$ ) were recorded at the final collection point at the end of each TT.

#### *Plasma $\text{NO}_3^-$ and $\text{NO}_2^-$*

Figure 5.1B shows plasma  $\text{NO}_3^-$  concentrations for all trials. The preloading  $\text{NO}_3^-$  dose administered ~6-10 h prior to the resting blood sample increased plasma  $\text{NO}_3^-$  concentrations in the CAFF+BJ and BJ trials ( $113.1 \pm 33.3$  and  $123.2 \pm 37.6 \mu\text{M}\cdot\text{L}^{-1}$  respectively;  $P < 0.01$ ) compared to the non-beetroot juice trials. Plasma  $\text{NO}_3^-$  levels remained significantly elevated in CAFF+BJ and BJ at all time points compared to CAFF and CONT ( $P < 0.05$ ). The second dose of beetroot juice (administered 130 min prior to the TT) further elevated plasma  $\text{NO}_3^-$  concentrations at 90 min ( $282.7 \pm 64.8$  and  $295.8 \pm 67.0 \mu\text{M}\cdot\text{L}^{-1}$ ) and 2 h post ingestion ( $310.6 \pm 58.7$  and  $333.9 \pm 64.7 \mu\text{M}\cdot\text{L}^{-1}$ ) compared to rest ( $P < 0.05$ ) with concentrations remaining elevated until after the TT ( $334.1 \pm 53.3$  and  $343.1 \pm 58.4 \mu\text{mol}\cdot\text{L}^{-1}$ ;  $P < 0.05$ ).

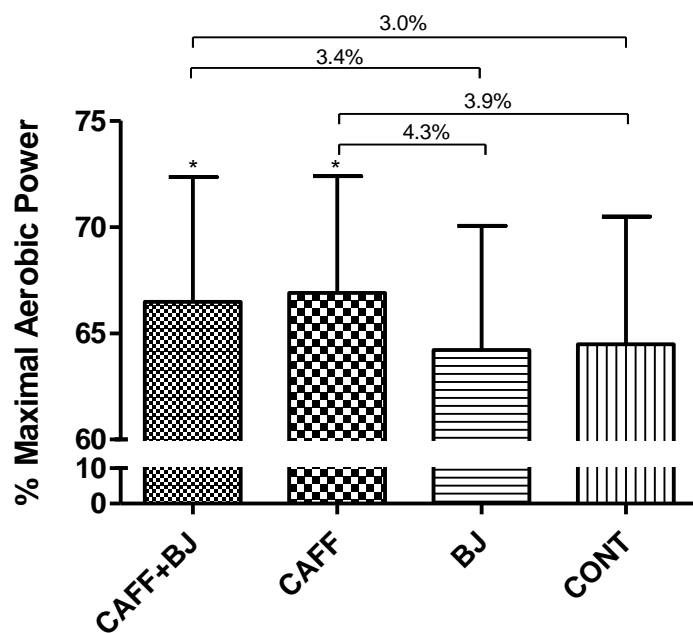
Figure 5.1C shows plasma  $\text{NO}_2^-$  concentrations for all trials. Concentrations were significantly higher in CAFF+BJ and BJ at all time points compared to CAFF and CONT ( $P < 0.01$ ). The resting blood sample reveals the preloading  $\text{NO}_3^-$  dose consumed ~6-10 h prior to the resting blood sample elevated  $\text{NO}_2^-$  levels to  $176.1 \pm 90.9$  and  $174.3 \pm 87.1 \text{ nmol}\cdot\text{L}^{-1}$  ( $P < 0.05$ ) for the CAFF+BJ and BJ trials respectively compared to the non-beetroot juice trials. The second dose of  $\text{NO}_3^-$  rich beetroot juice did not elevate plasma  $\text{NO}_2^-$  concentrations further in the CAFF+BJ and BJ trials.



**Figure 5.1.** Plasma caffeine, nitrate and nitrite concentrations; A) caffeine, B) Nitrate ( $\text{NO}_3^-$ ), C) Nitrite ( $\text{NO}_2^-$ ), CAFF+BJ (beetroot juice with caffeine); CAFF (caffeine); BJ (beetroot juice); CONT (placebo of caffeine and beetroot juice) (\$) different to CONT and BJ ( $P < 0.01$ ); (a) different to REST and 0 min ( $P < 0.01$ ); (b) different to REST, 0 min and 30 min ( $P < 0.05$ ); (c) different to REST; ( $\gamma$ ) different to CONT and CAFF ( $P < 0.01$ ); Times relative to ingestion; Rest in B and C include a ‘preload’  $\text{NO}_3^-$  dose ~6-10 h prior; Mean  $\pm$  SD.

### *Power output*

Figure 5.2 shows the relative mean power output combined for males and females. Caffeine improved mean power output compared to CONT in CAFF+BJ and CAFF trials on average by 3.5% ( $P < 0.01$ ). Beetroot juice supplementation had no effect on mean power output in either CAFF+BJ vs. CAFF or BJ vs. CONT. Using an inference based statistical approach caffeine was very likely (99%) and very likely (97%) (CAFF+BJ vs. BJ and CAFF vs. CONT respectively) to have a positive effect on performance outcomes during a cycling TT.  $\text{NO}_3^-$  supplementation was most unlikely (0%) and very unlikely (1%) (BJ vs. CONT and CAFF+BJ vs. CAFF respectively) to have any positive effect on performance. When mean power output was compared for trial order rather than intervention no significance was detected between any trial (Trial 1 through 4; Males,  $298 \pm 40$ ,  $301 \pm 35$ ,  $306 \pm 40$ ,  $305 \pm 37$  W respectively; Females,  $212 \pm 30$ ,  $210 \pm 26$ ,  $212 \pm 34$ ,  $208 \pm 31$  W respectively;  $P = 1.0$ ) indicating the Latin square design was successful in eliminating any possible trial order effect



**Figure 5.2.** Mean power output combined for males and females; CAFF+BJ (beetroot juice with caffeine); CAFF (caffeine); BJ (beetroot juice); CONT (placebo of caffeine and beetroot juice); (\*) different to CONT and BJ ( $P < 0.01$ ); Mean  $\pm$  SD.

### *Time trial completion time*

Times to complete the respective distances for males and females are presented in Table 5.1. For males when compared to CONT the time to complete the 43.83 km distance was reduced to a similar extent of 1.3% ( $P < 0.05$ ) for both the CAFF+BJ and CAFF trials. For females, the time to complete the 29.35 km distance was reduced by 0.9% and 1.6% ( $P < 0.05$ ) for the CAFF+BJ and CAFF trials respectively when compared to CONT. Beetroot juice supplementation had no significant positive or negative effect on time to complete the trials for both males and females in either CAFF+BJ vs. CAFF or BJ vs. CONT. Using an inference based statistical approach caffeine would possibly (65%) and likely (89%) for males and possibly (42%) and likely (88%) for females (CAFF+BJ vs BJ and CAFF vs. CONT respectively) produce a positive effect on performance outcomes during a cycling TT.  $\text{NO}_3^-$  supplementation was unlikely (7%) and very unlikely (1%) for males and very unlikely (0%) under both conditions for females (CAFF+BJ vs CAFF and BJ vs. CONT respectively) to have any positive effect on performance.

### *Heart rate and Rating of Perceived Exertion*

Table 5.1 shows mean heart rate and RPE for each trial for males and females. There were no differences in mean heart rate or RPE between any trials.

**Table 5.1.** Summary of cycling time trial performance and associated measures.

Intervention	Time	Power		Heart Rate	RPE	Body Mass	
	(h:mm:ss.00)	(W)	% MAP	bpm	6 - 20	Pre	kg Δ Pre-Post TT
Males	(43.83 km)						
CONT	1:03:30.39 ± 0:03:16.15	303 ± 41	66 ± 5.8	167 ± 11	17 ± 0.9	73.3 ± 6.7	-1.1 ± 0.4
CAFF+BJ	1:02:38.04 ± 0:03:31.00 *	314 ± 44 *	68 ± 6.3 *	171 ± 9	17 ± 0.9	73.6 ± 6.9	-1.4 ± 0.3
CAFF	1:02:43.86 ± 0:03:04.87 *	313 ± 38 *	68 ± 4.5 *	172 ± 10	17 ± 0.8	73.5 ± 7.0	-1.8 ± 0.3
BJ	1:04:05.03 ± 0:02:50.09	298 ± 35	65 ± 4.8	169 ± 9	17 ± 1.0	73.4 ± 6.9	-1.2 ± 0.3
Females	(29.35 km)						
CONT	0:51:40.10 ± 0:02:31.71	207 ± 29	63 ± 6.2	171 ± 8	17 ± 1.2	62.2 ± 8.9	-0.9 ± 0.4
CAFF+BJ	0:51:11.88 ± 0:02:22.13 *	212 ± 27 *	65 ± 5.1 *	176 ± 6	17 ± 0.8	62.0 ± 9.1	-0.9 ± 0.4
CAFF	0:50:50.53 ± 0:02:56.48 *	216 ± 34 *	66 ± 6.4 *	174 ± 9	17 ± 1.0	62.2 ± 9.0	-0.7 ± 0.3
BJ	0:51:41.06 ± 0:02:39.51	207 ± 31	63 ± 6.5	174 ± 9	17 ± 0.6	62.4 ± 9.3	-0.8 ± 0.5
Combined					% Δ (±90% CL)	p	
CONT	-	250 ± 57	64 ± 5.8	CAFF vs CONT	4.0 ± 1.7	< 0.001	
CAFF+BJ	-	258 ± 59	66 ± 5.6 *	CAFF+BJ vs CONT	3.1 ± 1.9	0.01	
CAFF	-	260 ± 58	67 ± 5.4 *	BJ vs CONT	-0 ± 1.3	0.6	
BJ	-	249 ± 56	64 ± 5.6	CAFF vs BJ	4.2 ± 1.8	< 0.001	
				CAFF+BJ vs BJ	3.4 ± 1.6	< 0.001	
				CAFF+BJ vs CAFF	-1 ± 1.7	0.4	

\* Significantly different to CONT and BJ (P < 0.05)

#### *5.4 Discussion*

This is the first study to determine the single and combined effects of caffeine and  $\text{NO}_3^-$  supplementation on the performance of cycling protocols that simulated real TT courses and were undertaken with the support of nutritional practices considered optimal for elite TT performance. As each of these ergogenic aids is purported to elicit their performance enhancing effect via different mechanisms (i.e., central vs. peripheral), it was hypothesised that the combination of the two interventions would increase mean power output to a greater extent than when each intervention was administered in isolation. The results indicate that caffeine supplementation provided a worthwhile enhancement of TT performance to both male and female cyclists, but that beetroot juice did not provide a detectable benefit under these conditions.

In the current study, pre-event supplementation with caffeine ( $3 \text{ mg} \cdot \text{kg}^{-1} \text{ BM}$ ) increased mean power output in cycling time trials lasting ~50 min (competitive female cyclists) and ~ 60 min (competitive male cyclists) to a similar extent (~3-4%) as reported previously using similar caffeine doses (Irwin et al., 2011, Jenkins et al., 2008, Lane et al., 2013a, Cox et al., 2002). In particular, these results are in agreement with the findings of Ryan et al. (2013) who reported that caffeine administered in the form of a gum prior to a cycling TT induced an elevation of circulating caffeine concentrations within 30 min of intake, and resulted in significantly improved performance. It was observed that the benefits of ingesting caffeine 40 min prior to time trials simulating the specific courses undertaken at the 2012 London Olympic Games were similar for males and females, although the courses they rode were slightly different in length and duration. Although these results were derived specifically for the preparation of cyclists for the 2012 Olympic Games, they can be generalised to other events of similar nature.

It is worth commenting that caffeine ingestion improved performance in this study under standardised conditions of dietary preparation that are both recommended and typical of the practices of cycling TT specialists. These practices included a CHO-rich pre-event meal (Lane et al., 2013b), consumption of a small fluid intake during the event according to the practical opportunities to drink (Garth and Burke, 2013) and frequent mouth contact with CHO (Lane et al., 2013b). Many studies often neglect to recognise that the real world application of ergogenic interventions may be influenced by optimal 'race day' strategies. Indeed, a meta-analysis has shown that the benefits of caffeine ingestion on endurance performance are reduced when it is taken in combination with CHO (Conger et al., 2011). However, under the conditions of this study caffeine ingestion still improved performance to the same degree as previously reported (Cox et al., 2002, Jenkins et al., 2008, Ryan et al., 2013, Irwin et al., 2011) even when guidelines for optimal CHO ingestion for this specific type of event (Burke et al., 2011) are followed. Lastly, the findings are also in agreement with Irwin et al. (2011) who reported a similar degree of improvement in cycling performance when a comparable ~12 h withdrawal from caffeine was enforced in habitual caffeine users. This observation suggests that longer withdrawal periods (24- 48 h) as previously recommended (Burke 2008) may not be necessary.

In contrast, no effect of beetroot juice ingestion on a cycling TT lasting ~50-60 min despite elevated plasma  $\text{NO}_3^-/\text{NO}_2^-$  concentrations was observed. Indeed, the conditions under which supplementation with  $\text{NO}_3^-$ /beetroot juice ingestion enhances exercise capacity or performance remain somewhat unclear. Elements that could be of importance include the timing and dose of  $\text{NO}_3^-$ , the intensity and duration of the exercise protocol, and the training history or calibre of the athlete. Recently the effect of beetroot juice on exercise capacity has been shown to be dose dependent, with the maximum benefits being seen with the acute ingestion of two bottles of beetroot juice concentrate (acute dose of 8.4 mM  $\text{NO}_3^-$ ) (Wylie et



al., 2013). Since the cyclists in this study ingested the same amount of the same product, both acutely (~2 h pre-exercise) and as an additional pre-load (6-10 h pre-trial), it is with confidence that the failure to detect benefits from  $\text{NO}_3^-$  supplementation cannot be explained by a sub-optimal dosing protocol.

The  $\text{NO}_3^-$  supplementation protocol substantially elevated plasma  $\text{NO}_2^-$  concentrations, although the peak values in this study were lower (225 vs. 470-687  $\text{nM}\cdot\text{L}^{-1}$ ) than those reported by studies employing a similar acute dosing protocol in subjects with a range of training histories (Wylie et al., 2013, Lansley et al., 2011a, Wilkerson et al., 2012, Cermak et al., 2012b, Muggeridge et al., 2013). Although only speculative, it is possible that the pre-race meal, consumed shortly before the ingestion of the second beetroot juice dose, may have affected the conversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ . However, despite this observed difference Cermak et al., (2012b) and Wilkerson et al., (2012) reported significantly higher peak plasma  $\text{NO}_2^-$  concentrations (532 and 472  $\text{nM}\cdot\text{L}^{-1}$  respectively) compared to the current study, but also failed to detect a performance benefit in well-trained cyclists. Due to the range of plasma  $\text{NO}_2^-$  concentrations, training histories as well as different performance tasks employed it is difficult to determine if these observations play a role in the effectiveness of  $\text{NO}_3^-$  supplementation.

The mechanism underpinning the observed benefits of  $\text{NO}_3^-$  supplementation on exercise capacity is believed to be a reduction in the oxygen cost of exercise, as a consequence of a reduced energy cost of contraction or enhanced mitochondrial efficiency (Jones et al., 2012). Whether this translates into an enhancement of performance across a range of exercise intensities has not been systematically studied. However, it is worth noting that the performance of shorter cycling tasks (~5-30 min duration) has been enhanced following  $\text{NO}_3^-$  supplementation. For example, in a study by Lansley et al. (2011a), subjects who sustained

intensities equivalent to ~98% and 95% of  $\dot{V} O_{2\max}$  during 4 km and 16.1 km time trials respectively recorded an improvement in performance after beetroot juice supplementation. However, a 50 mile cycling TT lasting ~135 min and eliciting a sustained exercise intensity equivalent to ~74% of  $\dot{V} O_{2\max}$  did not show a performance benefit following  $\text{NO}_3^-$  supplementation, despite subjects showing an improvement in power output per oxygen volume ( $\text{W/L}\cdot\text{min}^{-1}$ ) (Wilkerson et al., 2012). Cermak et al. (2012b) also reported no enhancement of 1 h cycling TT performance in a cohort of well-trained cyclists using a similar acute  $\text{NO}_3^-$  dose and timing strategy as employed in the current study. Although oxygen consumption was not measured during the TT in the current study, Coyle et al. (1991) reported that well-trained cyclists completed a 1 h TT at ~87%  $\dot{V} O_{2\max}$ , suggesting the current subjects of comparable straining status worked at a lower percentage of their aerobic capacity than those observed in shorter duration tasks as employed in the study of Lansley et al. (2011a). Possible explanations for this observation include the effects of exercise intensity on muscle oxygenation and motor unit recruitment. Higher exercise intensities are likely to result in a greater degree of skeletal muscle hypoxia, which would be expected to facilitate NO production through the reduction of  $\text{NO}_2^-$  (Maher et al., 2008). In addition, higher exercise intensities would be expected to mandate a greater recruitment of type II muscle fibres. There is evidence that the effects of  $\text{NO}_3^-$  supplementation on blood flow (Ferguson et al., 2013) and muscle force and calcium handling (Hernandez et al., 2012) might be more pronounced in type II fibres. These observations merit further investigation as it appears the effectiveness of  $\text{NO}_3^-$  supplementation may be influenced by the intensity of the performance task.

The failure to find a benefit of  $\text{NO}_3^-$  supplementation may be associated with the use of highly trained athletes due to a factor that is not currently identified. For example, studies in which pre-event ingestion of beetroot juice has been unable to produce a detectable

improvement in performance have involved sub-elite or well-trained cohorts (Cermak et al., 2012b, Muggeridge et al., 2013, Wilkerson et al., 2012, Christensen et al., 2013). A recent meta-analysis of studies of beetroot juice/nitrate supplementation and endurance performance published before August 2012 found that the effects were more readily observed in inactive to recreationally active individuals (Hoon et al., 2013). Clearly, this intriguing aspect warrants further study, with possible explanations including the optimisation of arginine-mediated pathways of NO production in highly-trained individuals or differences in muscle fibre-type (Christensen et al., 2013).

It is noteworthy that Cermak et al. (2012a) reported a significant improvement in 10 km cycle TT performance following 6 days of beetroot juice supplementation but no effect on 1 hour TT performance after acute beetroot juice intake (Cermak et al., 2012b). While this might be related to differences in exercise duration and intensity, as discussed earlier, it is also possible that longer periods of beetroot juice supplementation are necessary for performance changes to be realised in highly-trained subjects. For example, changes in proteins related to mitochondrial efficiency (Larsen et al., 2011) and muscle calcium handling (Hernandez et al., 2012) that have been reported following  $\text{NO}_3^-$  supplementation are likely to take several days (rather than hours) to become manifest.

Previous studies have suggested that there may be ‘responders’ and ‘non-responders’ to dietary  $\text{NO}_3^-$  supplementation (Wylie et al., 2013, Wilkerson et al., 2012, Christensen et al., 2013) and this observation appears to be consistent within a highly trained cohort (Christensen et al., 2013, Wilkerson et al., 2012). In the current study only two male individuals recorded better performances in both BJ vs CONT and CAFF+BJ vs. CAFF, suggesting they were possible ‘responders’. In comparison Christensen and co-workers (2013) noted that two of the 10 highly trained cyclists in their study (mean aerobic capacity

of 72.1 mL.kg<sup>-1</sup>.min<sup>-1</sup> vs. 71.6 mL.kg<sup>-1</sup>.min<sup>-1</sup> in the male cyclists in the current study) appeared to benefit from a chronic beetroot juice intake protocol, deriving a ~3% improvement in performance of an ~18 min TT compared with a control condition. Factors to explain individual responsiveness to such supplementation remain elusive at present.

In conclusion this study provides evidence that a caffeine gum containing 3 mg.kg<sup>-1</sup> BM ingested in the 40 min prior to a cycling TT lasting ~45-60 min increases cycling power output in both males and females. However, despite increasing circulating NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> concentrations beetroot juice supplementation ingested ~8-12 h prior as well as an acute dose ingested ~2 h prior to the TT did not enhance cycling performance either in isolation or in combination with caffeine ingestion. Based on previous evidence that NO<sub>3</sub><sup>-</sup> supplementation can improve performance under a variety of high intensity endurance tasks it cannot be ruled out that the possibility of an additive effect may still be possible with different protocols or to specific individuals ('responders'). Further research is required to determine if NO<sub>3</sub><sup>-</sup> supplementation can further enhance performance when co-ingested with caffeine under shorter more intense tasks where the benefit of NO<sub>3</sub><sup>-</sup> supplementation is more pronounced.

### *5.5 Acknowledgments*

The hard work and commitment of the athletes who gave their time to participate in this research project was greatly appreciated. This study would also not have been possible without the assistance of the physiology and sports nutrition staff at the Australian Institute of Sport who assisted in running the trials. Ben Desbrow is acknowledged for his contribution for the analysis of plasma caffeine concentrations as well as Andrew Jones and James Blackwell for the analysis of plasma nitrate/nitrite concentrations.

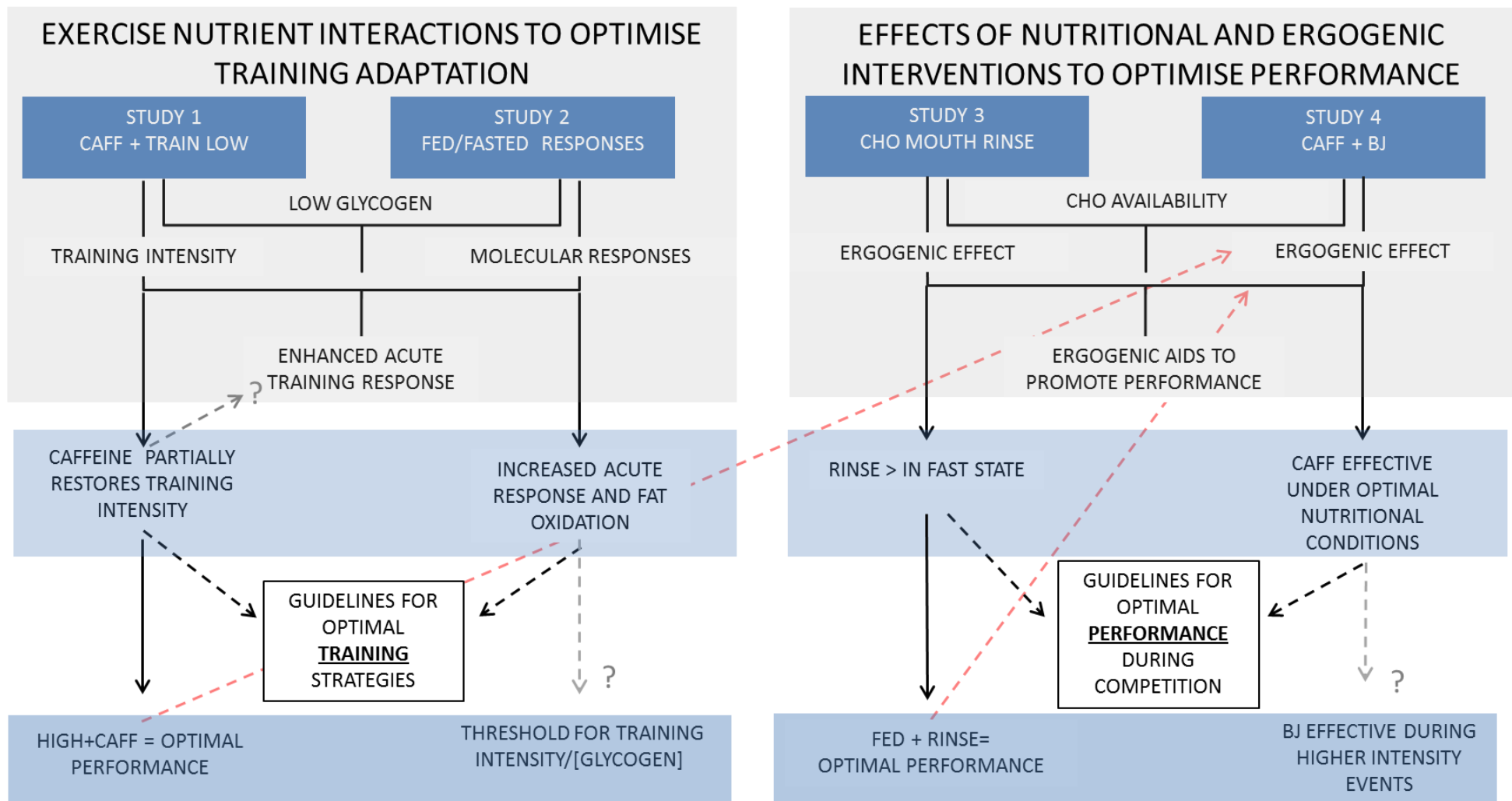


# ***Chapter Six***

## ***Summary and conclusions***

The main aims and major findings for the studies undertaken for this thesis, as well as the incorporation of these findings into subsequent investigations are shown schematically in Figure 6.1. The general aims of the studies undertaken as part of this thesis were twofold. Firstly, Chapters two and three (study one and two; figure 6.1, left panel) investigated exercise-diet interventions to enhance training adaptation under conditions of reduced muscle glycogen availability. Specifically, these studies investigated protocols aimed to restore and or maintain training intensity under conditions of reduced muscle glycogen concentrations, as well as the effects of the timing of CHO feeding in relation to exercise on acute training responses. The findings of these studies provide insight for the development of guidelines to promote training adaptation under conditions of reduced CHO availability (Figure 6.1, left panel, black dashed lines) as well as directions for future research (Figure 6.1, grey dashed lines). A second aim was to determine the effects of selected ergogenic supplements under conditions of optimal or sub-optimal CHO availability with the intent to provide evidence for their efficacy during competition in situations where athletes follow current sports nutrition guidelines (Chapters four and five; Figure 6.1, right panel). In the development of protocols for nutritional/ergogenic practices that support optimal performance during competition, the specific findings from the early studies of this thesis were subsequently incorporated into the design of the final investigations (Figure 6.1, red dashed lines).





**Figure 6.1.** Major findings of each study and the incorporation of guidelines for optimal performance during subsequent investigations.

The first study (Chapter two) investigated the effects of caffeine ingestion on cycling power output when HIT was commenced with reduced or normal muscle glycogen concentrations. It was hypothesised that caffeine ingested prior to an exercise bout commenced with reduced glycogen availability would 'rescue' the previously observed decline in training intensity commensurate to that when exercise was commenced with normal resting glycogen concentrations. The main findings of this study were that independent of glycogen availability, a low dose of caffeine ( $3 \text{ mg} \cdot \text{kg}^{-1} \text{ BM}$ ) enhanced work capacity during intense interval training by approximately 3%. In contrast, commencing HIT with reduced glycogen availability reduced mean power output by ~8%. It was concluded that the ergogenic effect of caffeine was insufficient to completely 'rescue' the decrease in power output observed when undertaking high-intensity, aerobic-based training with low muscle glycogen availability.

The enhancement in power output observed after caffeine ingestion during HIT commenced with low glycogen availability, provides a basis for future studies to investigate if caffeine can further enhance the already augmented response observed when selected sessions are commenced with low glycogen levels. Future research needs to test this hypothesis in studies investigating the acute molecular training response as well as in long-term training studies to determine if it results in enhanced adaptation and performance. Additionally, the findings of this study reinforce the viewpoint that low doses of caffeine are capable of significantly improving performance during competition under conditions of both high and low glycogen availability.

The results from Study one demonstrated that caffeine could not fully restore the decline in training intensity during HIT. Another possibility that may still take advantage of the augmented 'train-low' response while preserving overall training intensity is to complete HIT while glycogen levels are high and then complete a subsequent low intensity aerobic session

with reduced glycogen content. In this approach the intensity of a second steady-state training bout would presumably be less likely to be compromised as a result of reduced glycogen availability. As such, Study two (Chapter three) investigated the effects of withholding feeding post an evening HIT bout and sleeping with reduced glycogen content, and the effects of commencing a subsequent low intensity exercise bout with reduced glycogen concentrations on acute signaling and gene responses during recovery.

In this study the timing of meals ingested throughout the day was designed so that in one trial all meals were consumed prior to an evening session of HIT (FASTED), while in a second trial a high CHO meal was ingested immediately post-HIT (FED). Importantly, the total daily energy intake on the day of the experimental trial was the same with only the timing of ingestion of meals different. This ‘train-high, sleep-low’ approach offers a practical strategy to ensure an athlete’s daily energy intake targets are met while providing a practical way to delay feeding post-exercise with the intent of promoting adaptation to training. The initial hypothesis was that by withholding feeding during recovery, the combined stimulus of reduced glycogen and the concomitant increase in FFA concentrations would increase and prolong the acute adaptive response. The results partially supported this hypothesis in that by restricting feeding post-HIT in the FASTED trial the phosphorylation of AMPK, p38MAPK and p-ACC were elevated to a greater extent ~8 h later at rest the following morning compared to when a high CHO meal was consumed immediately post-HIT. In addition to the partial restoration of glycogen stores in the FED trial, the differences in cellular responses observed may also be attributed to differences in systemic factors such as elevated insulin and glucose concentrations, or alternatively, the sustained elevation of plasma FFA concentrations in the FASTED trial. In contrast to the original hypothesis the protein and mRNA content for specific downstream targets of AMPK, p38MAPK failed to show the same temporal pattern of activation. An explanation for this ‘mismatch’ could be that the

magnitude of change in the activity of AMPK and p38MAPK was insufficient to mediate changes in downstream targets, or that other stimuli are required to evoke such responses. Alternatively, due to high variability in the timeline for gene responses and the subsequent accumulation of mRNA and proteins it is possible that the timing of the biopsies failed to detect peak mRNA concentrations and the 'snap shot' fails to capture the entire picture with regard to intracellular signaling events.

A second hypothesis of this study was that by commencing a low intensity aerobic exercise bout the following morning after withholding feeding overnight, molecular markers of adaptation would be upregulated to a greater extent in FASTED when compared to the FED trial. This study was designed to provide insight as to whether a lower intensity exercise bout could augment the training response in the face of low glycogen to the same extent as that previously observed after high intensity training. The aerobic bout failed to further elevate markers for mitochondrial biogenesis in both trials, suggesting the exercise intensity was insufficient to stimulate this response, or that the response had already reached its maximal threshold.

Commencing exercise with reduced glycogen content in the FASTED trial elevated several markers for lipid transport and oxidation with subsequently higher rates of fat oxidation recorded during the steady-state ride. Substrate utilisation during submaximal exercise up to intensities of ~65% of  $\dot{V} O_{2\max}$  is predominantly supplied from circulating FFA and intramuscular triglycerides. This study has shown that withholding feeding post-exercise and commencing a subsequent steady-state training bout with reduced glycogen availability has the capacity to further promote the upregulation of lipid transport and oxidation. Future investigations now need to determine if there is possibly a lower 'threshold' of exercise intensity that is required to promote beneficial adaptations when exercise is commenced with

reduced glycogen concentrations. Similarly, future studies should also aim to determine if a 'critical' level of glycogen depletion is required in order to evoke the previously observed enhanced training response when exercise is commenced with reduced glycogen concentrations.

Study three (Chapter 4) determined the effects of a CHO mouth rinse on cycling time trial performance commenced in a fed or fasted state. This investigation aimed to clarify the conflicting observations of previous research that collectively suggested that the efficacy of the mouth rinse procedure was less pronounced when a 'pre-event' meal was ingested in the hours prior to a performance bout. Accordingly, it was hypothesised that a CHO mouth rinse would be less effective when a CHO rich meal was ingested 2 h prior to a 60 min cycling time trial compared to when the same trial was commenced in an overnight fasted state. Results confirmed this hypothesis, with the enhancement in performance greater when undertaken in the fasted state (3.4%) than compared to the fed state (1.8%). The findings also showed that consuming a CHO-rich meal ( $2.5 \text{ g}\cdot\text{kg}^{-1} \text{ BM}$ ) two hours prior to a time trial enhances mean power output compared to undertaking the time trial in a fasted state. Results of this study provide insight for the development of future recommendations to athletes regarding pre-event feeding. For athletes who refrain from ingesting a meal prior to high intensity exercise due to potential gastrointestinal (GI) problems, the results suggest that performance will be reduced compared to consuming a pre-event meal. However, the use of a CHO mouth rise (which could avoid GI problems) can improve performance by a magnitude equal to the effect of consuming the pre-event meal. The best performance outcome however, appears to be achieved by the combination of both a CHO-rich pre-event meal and the regular use of a CHO mouth rinse throughout a time trial. This observation was incorporated into the design of study 4 (Chapter 5; shown in Figure 6.1) where the objective was to investigate the effectiveness of ergogenic aids under conditions of optimal CHO availability

The final study (Chapter Five) determined the effects of two well-known ergogenic aids, caffeine and beetroot juice, on a performance trial replicating the demands of the 2012 London Olympic cycling time trial course. The novelty of this study was the assessment of any added benefit to performance if both supplements were co-ingested, and because of the experimental design (Latin squares), it was also possible to determine the benefit of each ergogenic aid in isolation. Few studies have explored the possible combined effects of ergogenic aids despite the rationale that if each acts through a different (independent) mechanism an additive performance improvement may be observed. Specific to this study the well accepted centrally mediated effect of caffeine (shown to enhance performance regardless of CHO availability; see Figure 6.1) combined with the improvement in metabolic efficiency afforded by dietary  $\text{NO}_3^-$  provided a potential combination. Since the development and completion of this study a collection of related literature has since been published. This literature has provided further evidence for factors that may influence the ergogenic benefit of  $\text{NO}_3^-$  supplementation. The findings that beetroot juice had no effect on performance under the experimental conditions were at the time unexpected. The growing body of literature now provides insight into aspects of exercise duration and intensity of the performance trials as well as the training status of the athletes. The lack of effect of beetroot juice supplementation in this study (as well as recent investigations that have also failed to detect a performance benefit) may be related to these more recent observations. It is possible that the reduction in oxygen cost observed after  $\text{NO}_3^-$  supplementation may be more beneficial during shorter more intense events in the range of 3-15 min where the fractional utilisation of  $\dot{V} \text{O}_{2\text{max}}$  exceeds >85%. It is also possible that the well-trained subjects recruited in this study did not 'respond' to the  $\text{NO}_3^-$  supplementation protocol employed. It has been suggested that well trained subjects, for reasons that include better metabolic regulation and elevated resting  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations, may require higher doses or longer loading regimes to elicit

similar ergogenic effects to those observed in untrained subjects. Lastly, the findings of this study further add to the previous results of study one (Chapter Two) with caffeine again being demonstrated to have a robust effect on performance even under conditions of optimal race day nutritional strategies.

In conclusion, the experiments undertaken for this thesis add novel information to our current understanding of nutritional and ergogenic strategies that can promote adaptation to exercise training, as well as enhance performance during competition. Specifically, the current body of work has investigated two strategies to enhance training adaptation under conditions of reduced glycogen availability. The results provide evidence that the use of ergogenic supplements during training under conditions of low glycogen availability can enhance training intensity, but not to levels seen when athletes commence the same sessions with high CHO availability. Although, the robust performance enhancing effects of caffeine have been repeatedly shown in Chapters two and five. A novel ‘train-high, sleep-low’ approach, which combines exercise nutrient strategies to promote training adaptation, has been experimentally tested, with results suggesting that adaptive mechanisms may be sensitive to critical thresholds of glycogen concentrations as well as training intensity. Results from the experiments undertaken for this thesis also provide evidence for the performance enhancing effects of select ergogenic strategies used during competition commenced under nutritional conditions typical of elite athletes.

Collectively, the findings from these studies provide insight into novel exercise-nutrient strategies to promote training adaptation, as well as ergogenic strategies to optimise performance during competition. The results of the work undertaken for this thesis provide a scientific rationale on which future research in the areas of exercise-nutrient interactions can be based.





# ***Chapter Seven***

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## ***Appendix I***





## ***Appendix II***



## ***Appendix III***